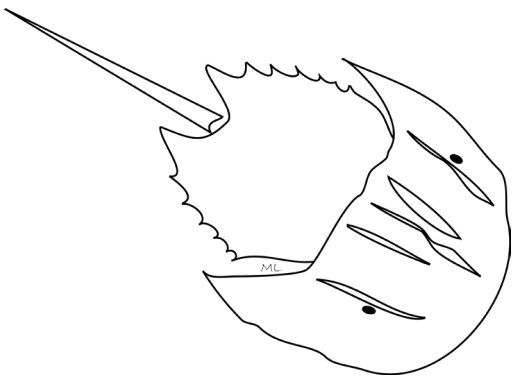
DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

29/2016

MARIANN LASSENIUS

Bacterial Endotoxins in Type 1 Diabetes



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Bacterial Endotoxins in Type 1 Diabetes

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ACADEMIC DISSERTATION

To be presented, with the permission of the Medical Faculty of the University of Helsinki,
for public examination in Lecture Hall 2, Biomedicum Helsinki,
on June 10th 2016, at 12 noon

Helsinki 2016

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ISBN 978-951-51-2103-5, ISSN 2342-3161 (paperback)
ISBN 978-951-51-2104-2, ISSN 2342-317X (pdf)
http://ethesis.helsinki.fi
Hansaprint Oy
Helsinki 2016

1. Contents

2. List of original publications

This thesis is based on the following publications:

I. Nymark M, Pussinen P, Tuomainen A, Forsblom C, Groop PH, Lehto M. Serum lipopolysaccharide (LPS) activity is associated with the progression of kidney disease in Finnish patients with type 1 diabetes. *Diabetes Care*, 2009, 32(9):1689-93

II. Lassenius MI, Pietiläinen K, Kaartinen K, Pussinen P, Syrjänen J, Forsblom C, Pörsti I, Rissanen A, Kaprio J, Mustonen J, Groop PH, Lehto M. Bacterial endotoxin activity in human serum is associated with dyslipidemia, insulin resistance, obesity, and chronic inflammation. *Diabetes Care*, 2011, 34(8):1809-1815

III. Lassenius MI, Mäkinen VP, Fogarty CL, Peräneva L, Jauhiainen M, Pussinen PJ, Taskinen MR, Kirveskari J, Vaarala O, Nieminen JK, Hörkkö S, Kangas AJ, Soininen P, Ala-Korpela M, Gordin D, Ahola AJ, Forsblom C, Groop PH, Lehto M, on behalf of the FinnDiane Study Group. Patients with type 1 diabetes show signs of vascular dysfunction in response to multiple high-fat meals. *Nutrition and Metabolism,* 2014 Jun 13;11:28

IV. Lassenius MI, Fogarty CL, Blaut M, Haimila K, Riittinen L, Paju A, Kirveskari J, Järvelä J, Ahola AJ, Gordin D, Kumar A, Hamarneh S, Hodin R, Hörkkö S, Pussinen P, Forsblom C, Jauhiainen M, Taskinen MR, Groop PH, and Lehto M on behalf of the FinnDiane Study Group. Intestinal alkaline phosphatase at the crossroad of intestinal health and disease: a putative role in type 1 diabetes. *Submitted.*

3. Abbreviations

AER - albumin excretion rate
AGE - advanced glycation end product
AHT – anti hypertensive medication
AIx - augmentation index
AOAH - acyloxyacyl hydrolase
AP - alkaline phosphatase
Apo - apolipoprotein
ATP - adenosine triphosphate
AUC - area under the curve
BMI - body mass index
CD14 - cluster of differentiation 14
CETP - cholesteryl ester transfer protein
CIAP - calf intestinal alkaline phosphatase
CKD - chronic kidney disease
CRP - C - reactive protein
CuOx-LDL - cupper oxidized LDL
CV - coefficient of variation
CVD - cardiovascular disease
DAG - diacylglycerol
DCCT - Diabetes Control and Complications Trial
DN - diabetic nephropathy
EDIC - Epidemiology of Diabetes Interventions and Complications Research Group
eGDR - estimated glucose disposal rate
eGFR - estimated glomerular filtration rate
ELISA - enzyme linked immunosorbent assay
eNOS - endothelial nitric oxide
ESRD - end-stage renal disease
EU - endotoxin units
FUT2 - fucosyltransferase 2
GAPDH - glyceraldehyde phosphate dehydrogenase
GSH - glutathione
HbA_{1C} - glycated hemoglobin
HDL - high density lipoprotein
HMGB1 - high-mobility group box 1
HOMA-IR - homeostasis model of insulin resitance
HSP - heat shock protein
HUSLAB - Helsinki University Hospital laboratory
IAP - intestinal alkaline phosphatase
IBD - inflammatory bowel disease
Ig - immuloglobulin
IgAGN - IgA glomerulonephritis
IL - interleukin
IncA - incremental area under the curve
IU - insulin units
LADA - latent autoimmune diabetes of adults
LAL assay - limulus amebocyte lysate assay
LBP - LPS binding protein
LDL - low density lipoprotein
LPS - lipopolysaccharide
LRP1 - lipoprotein-receptor related protein 1
LTA - lipoteichoic acid
MAA-LDL - malondialdehyde acetaldehyde LDL
MCP-1 - monocyte chemoattractant protein 1
MD-2 - myeloid differentiation 2
MODY - maturity onset diabetes of the young
NADPH - nicotinamide adenine dinucleotide phosphate
NDC - non-diabetic control
NMR - nuclear magnetic resonance
NOD mice - non-obese diabetic mice
oxLDL – oxidized LDL
PAI-1 - plasminogen activator inhibitor
PARP - poly (ADP-ribose) polymerase
PDK-1 - 3-phosphoinositide-dependent kinase-1
PKC - protein kinase C
PLTP - phospholipid transfer protein
pNPP - p-nitrophenyl phosphate
PON1 - paraoxonase
RAGE - receptor for AGE
RNA - ribonucleic acid
RAS – renin-angiotensin system
SAA - serum amyloid A
SCFA – short-chain fatty acids
T1D - type 1 diabetes
T2D - type 2 diabetes
TGF-β – transforming growth factor β
TLR - toll-like receptor
uCrea - urinary creatinine
uPAR - urokinase plasminogen activator receptor
VEGF – vascular endothelial growth factor

WHO - World Health Organization
WHR – waist-to-hip ratio
VLDL - very-low density lipoprotein

4. Abstract

Background
Around one-third of patients with type 1 diabetes develop diabetic nephropathy. Even though the pathogenesis of diabetic nephropathy is not fully understood, the process involves several environmental factors. Low grade inflammation has been linked to many metabolic diseases and is also evident in patients with type 1 diabetes, especially in the presence of nephropathy. Bacterial lipopolysaccharides (LPS) are powerful triggers of inflammation, but whether low grade inflammation is caused by these components is an open question.

Aims
To examine the relationship between LPS and the development of diabetic nephropathy, inflammation, vascular function, lipid metabolism, and intestinal homeostasis in patients with type 1 diabetes.

Subjects and methods
These studies are part of the ongoing Finnish Diabetic Nephropathy Study (FinnDiane), a nationwide, multicenter study that aims to identify genetic and environmental risk factors for the development of diabetic complications in patients with type 1 diabetes. Study I was a follow-up study, and Studies II–IV were cross-sectional in their design. Renal status was verified at follow-up by laboratory data and a review of all available medical files (Study I). Study II included, in addition to patients with type 1 diabetes, patients with IgA glomerulonephritis and non-diabetic control subjects. The participants in Studies III and IV had three consecutive high-fat meals and were followed for 10 hours postprandially. Factors associated with endotoxemia, vascular function, inflammation, and lipid metabolism were investigated. For all studies, LPS was measured by the limulus amebocyte lysate (LAL) assay from serum samples.

Results
In the patients with type 1 diabetes, high serum LPS activity at baseline was associated with the development of microalbuminuria during the follow-up. High serum LPS was also associated with features of the metabolic syndrome in both the patients with type 1 diabetes and the overweight non-diabetic controls. Of note, no accumulation of LPS in the circulation was evident in response to three high-fat meals. However, the patients with type 1 diabetes showed altered postprandial lipid metabolism and vascular response. Moreover, factors associated with gut homeostasis were altered in the patients with type 1 diabetes compared to the non-diabetic controls.

Conclusions
We show that high serum LPS is associated with the development of diabetic nephropathy and features of the metabolic syndrome. The metabolic syndrome is associated with insulin resistance, and it is a risk factor for both diabetic nephropathy and cardiovascular disease. Endotoxins may affect the development of diabetic nephropathy through the direct disruption of the filtration barrier, but also through insulin resistance at both the tissue and systemic levels. In response to acute high-fat feeding, no evidence for LPS accumulation was seen. However, unfavorable changes in lipid metabolism and vascular response in patients with type 1 diabetes may render them at higher cardio-metabolic risk. This risk may

be further enhanced by adverse changes in both inflammatory and protective factors in the gut, leading to a possibly higher gut permeability and an increase in circulating endotoxins.

Abstract in Finnish

Noin kolmasosalle tyypin 1 diabetesta sairastaville potilaille kehittyy diabeettinen munuaistauti. Vaikka tautikuvaan liittyvää patologiaa ei ymmärretä täysin, monet ympäristötekijät vaikuttavat taudin kehitykseen. Matala-asteisen tulehduksen on osoitettu olevan yhteydessä aineenvaihdunnallisiin tauteihin, niin myös tyypin 1 diabetekseen, etenkin munuaistaudin ollessa läsnä. Bakteeriperäiset lipopolysakkaridit (LPS, endotoksiinit) ovat voimakkaita tulehdusvastetta käynnistäviä molekyylejä. Endotoksiinien merkitys elimistön pitkittyneissä tulehdustiloissa on kuitenkin ollut toistaiseksi epäselvä.

Väitöskirjani tarkoituksena oli tutkia LPS:n osuutta diabeettiseen munuaistautiin, tulehdukseen, verisuonten toimintaan, rasva-aineenvaihduntaan sekä suoliston tasapainoon tyypin 1 diabeetikoilla.

Nämä tutkimukset ovat osa käynnissä olevaa Finnish Diabetic Nephropathy Study (FinnDiane) tutkimusta, joka on maankattava monikeskustutkimus. FinnDiane tutkimuksen tavoitteena on löytää geneettisiä- ja ympäristötekijöitä, jotka vaikuttavat diabeettisen munuaistaudin kehittymiseen. Osatyö I oli seurantatutkimus ja osatyöt II-IV poikkileikkaustutkimuksia. Munuaisten tila määritettiin seuranta-aikana käyttäen laboratoriotutkimusten tuloksia ja muita potilastietoja (osatyö I). Osatyössä II, diabeetikoiden lisäksi tutkimme IgA glomerulonefriittiä sairastavia potilaita sekä ei-diabeettisia verrokkeja. Osatöihin III ja IV osallistuvat, saivat kolme perättäistä runsasrasvaista ateriaa, jonka jälkeen heitä seurattiin 10 tuntia. Endotoksemiaan (LPS), verisuonten toimintaan, tulehdukseen ja rasva-aineenvaihduntaan liittyviä tekijöitä määritettiin aterianjälkeisistä näytteistä. Kaikissa osatöissä seerumin LPS aktiivisuus määritettiin limulus amebosyytti lysaatti (LAL) menetelmällä.

Seurantatutkimuksessa, lähtötilanteen korkea seerumin LPS aktiviteetti lisäsi tyypin 1 diabeetikoiden munuaisvaurion/mikroalbuminurian kehittymisen riskiä. Korkea seerumin LPS aktiivisuus liittyi myös metabolisen oireyhtymän piirteisiin sekä tyyppi 1 diabetes potilailla että ylipainoisilla ei-diabeettisilla verrokeilla. Päivän kestävässä rasvarasituksessa ei pystytty suoraan osoittamaan verenkierron LPS-aktiivisuuden lisääntymistä. Aterioiden jälkeen tyypin 1 diabetes potilailla oli kuitenkin epäsuotuisia muutoksia rasva-aineenvaihdunnassa ja verisuonten toiminnassa. Lisäksi diabeetikoilla havaittiin epäsuotuisia muutoksia suoliston tulehduksellista tilaa ilmentävissä tekijöissä.

Tutkimuksemme osoittaa, että LPS vaikuttaa diabeettisen munuaistaudin kehitykseen ja metabolisen oireyhtymän piirteisiin. Metabolinen oireyhtymä on suoraan yhteydessä insuliiniresistenssiin, mikä itsessään on tunnettu riskitekijä sekä diabeettisessa munuaistaudissa että sydän- ja verisuonitaudeissa. Endotoksiinit voivat suoraan vaikuttaa munuaisen toimintaan lisäämällä munuaiskeräsen läpäisevyyttä proteiineille, mutta myös epäsuorasti insuliiniresistenttiyden kautta sekä kudostasolla. Lyhytkestoisen rasvarasituksen ei todettu suoraan vaikuttavan LPS:n kertymiseen verenkiertoon. Epäsuotuisat muutokset rasva-aineenvaihdunnassa ja verisuonten vasteessa saattavat kuitenkin lisätä tyypin 1 diabeetikoiden alttiutta kardio-metabolisille sairauksille. Epäedulliset muutokset suoliston suojaavissa tekijöissä voivat muuttaa mikrobiflooran koostumusta ja lisätä suolen seinämän läpäisevyyttä, mikä puolestaan voi lisätä bakteerien aiheuttamia tulehduksia tyypin 1 diabeetikoilla.

5. Introduction

The discovery of insulin by Banting and Best in the 1920s and its industrial production saved many thousands of lives of people with type 1 diabetes [1]. In just a few decades, people with type 1 diabetes were able to live long enough to develop complications. Changes in the kidneys of patients with type 1 diabetes were described as early as 1936 by Paul Kimmelstiel and Clifford Wilson [2]. Since then, evidence has accumulated that emphasizes the role of strict glycemic control for preventing diabetic complications, improving life expectancy, and for decreasing the occurrence of complications. Yet diabetic nephropathy is estimated to affect about one-third of patients with type 1 diabetes [3,4]. The pathogenesis of diabetic nephropathy is still poorly understood, but alongside genetic propensity, it involves a large number of environmental factors [5].

Chronic low grade inflammation has been associated with obesity, cardiovascular disease, and type 2 diabetes [6,7]. Low grade inflammation (CRP <10 mg/l) is also evident in patients with type 1 diabetes, especially in those with diabetic nephropathy [8]. Whether the low grade inflammation is caused by exposure to bacteria or bacterial components is still not clear.

Our environment is filled with beneficial and detrimental bacteria that influence our health. In the search for factors that trigger inflammation, a potent pro-inflammatory molecule, lipopolysaccharide (LPS), which is a structural part of the cell membrane of gram-negative bacteria, has become the focus of interest in the research on chronic inflammation.

Notably, under laboratory conditions in rodent models, LPS has long been used to induce kidney injury [9-11], raising the possibility that LPS also affects inflammation and kidney function in humans. In the human intestine alone, microorganisms exceed the cells of our body tenfold and are a source of microbial components [12,13]. This represents a large pool of LPS that can gain access to the circulation through passive leakage or direct uptake together with dietary fats.

The present studies were conducted to evaluate the effect and source of bacterial endotoxins on inflammation in patients with type 1 diabetes, and the impact on the development of diabetic nephropathy.

6. Review of the literature

6.1. Diabetes

The global prevalence of diabetes was 9% among the adult population in 2014, corresponding to 347 million people worldwide, and approximately 1.5 million deaths were estimated to have been directly caused by diabetes (WHO website). Global projections estimate that by 2035 the number of people with diabetes will increase by 55%, affecting 592 million people, thereby posing an unacceptably high human, social, and economic burden on countries at all income levels (IDF, Diabetes atlas webpage 2.9.2015).

Diabetes is a group of metabolic disorders that share a common feature, elevated blood glucose. Traditionally, this condition has been separated into two entities: type 2 diabetes (T2D), where the tissues are unable to respond normally to insulin, and type 1 diabetes (T1D), where the insulin-producing cells of the pancreas are destroyed in an autoimmune process, leading to a lifelong dependence on external insulin [14]. However, contrary to being dichotomous disorders, T1D and T2D rather represent opposite ends of a spectrum within which intermittent forms of diabetes also exist: gestational diabetes that usually resolves postpartum; impaired glucose tolerance with slightly elevated fasting or postprandial glucose concentrations (also called pre-diabetes); maturity onset diabetes in the young (MODY), characterized by an autosomal dominant inheritance, an early onset of diabetes, and mild hyperglycemia; and the latent autoimmune diabetes of adults (LADA), which represents a slowly progressing autoimmune diabetes. Moreover, in T2D insulin production will eventually also decrease, as in T1D [14].

The treatment of diabetes and its complications poses an increasing burden on the healthcare system. The costs of treating patients with diabetes have been estimated to consume about 11 to 12% of the total health care costs in Finland [15]. In 2007 the health care expenditure for uncomplicated T1D was 41.5 million euros, and the presence of complications more than doubled the costs to 135 million euros [16]. The indirect costs of diabetes further arise from loss of productivity due to premature morbidity and mortality, and these costs were 1,133 million euros in 2007 [16]. Preventing diabetic complications is thus imperative not only for the quality of life of the patients but also from the perspective of the national economy.

T1D is an autoimmune disease, characterized by absolute insulin deficiency caused by the destruction of pancreatic β-cells [14]. Activation of the immune system by triggering environmental factors leads to an inflammatory response causing β-cell autoimmunity/insulitis, the appearance of autoantibodies, the progressive loss of β-cells, and finally clinical disease [14]. The worldwide incidence of T1D in children has been increasing over the past 50 years [17]. Over the past decades, it has varied from 0.1 in Venezuela to 60 per 100,000 persons per year in Finland, the country with the highest incidence rates in the world [18-20]. However, the increase in new cases of T1D in Finland seems to have leveled off in the past 10 years [20].

The pathogenesis of T1D is not fully understood. Apart from genetic risk factors, environmental factors associated with disease incidence include viral infections, toxins, early exposure to complex dietary proteins, high body weight, insulin resistance, and intestinal inflammation [21]. The factors causing T1D are complex, and gene–gene, gene–environment, and environment–environment interactions probably occur during the disease process [21].

6.2. Complications in type 1 diabetes

Diabetes is associated with an increased risk of microvascular and macrovascular disease. Microvascular diseases encompass nephropathy, retinopathy, and neuropathy. Macrovascular complications include cardiovascular diseases such as atherosclerosis, myocardial infarction, and stroke [22]. The studies involved in this thesis focus on environmental risk factors and their association with diabetic complications [23]. Modifiable risk factors for diabetic complications include hypertension, dyslipidemia, smoking, and poor glycemic control. Clinically, the use of glycated hemoglobin (HbA_{1C}) in the evaluation of long-term glucose control is important, as the HbA_{1C} levels reflect the past 8 to 12 weeks of average blood glucose [24]. However, additional factors contributing to the risk of diabetic complications have recently been identified, including chronic low grade inflammation, advanced glycation end products, and a lack of physical activity [5].

6.2.1. Diabetic nephropathy

Despite the constantly improving care, diabetic nephropathy (DN) is estimated to affect one-third of patients with T1D [3]. DN is strongly associated with cardiovascular mortality. End-stage renal disease (ESRD) is associated with an 18-fold increased risk of premature mortality compared to non-diabetic individuals of the same age and sex [25,26].

Healthy kidneys filter more than one liter of blood per minute. Their main function is to maintain body homeostasis by dealing with metabolic waste products, water, and electrolytes. Other functions of the kidney include blood pressure regulation, the regulation of erythropoietin, and the regulation of calcium homeostasis.

Urinary filtration takes place in the functional unit of the kidney, namely the nephron. The filtration barrier consists of four layers: the glycocalyx, which by its negative charge repels many proteins; the endothelium; the glomerular basement membrane; and the podocytes (Figure 1) [27]. Blood enters the glomerulus through the afferent arteriole, and the high pressure in the capillaries causes water and small soluble molecules to move through the fenestrated endothelial cells, glomerular basement membrane, and the slit diaphragm formed by the podocytes into the proximal tubule (Figure 1). Mesangial cells give structural support to the glomerulus and regulate its blood flow by vascular constriction of the capillaries. From the primary urine, water and other important molecules are reabsorbed [27]. Since the endothelium, podocytes, and tubulointerstitial cells that lie between the tubules are tightly interconnected, dysfunction can spread from one compartment to the other during the development of DN. The pathological processes include foot process effacement of the podocytes, loss of the negatively charged glycocalyx, and tubulointerstitial fibrosis leading to an increase in urinary proteins and decreased filtration [27].

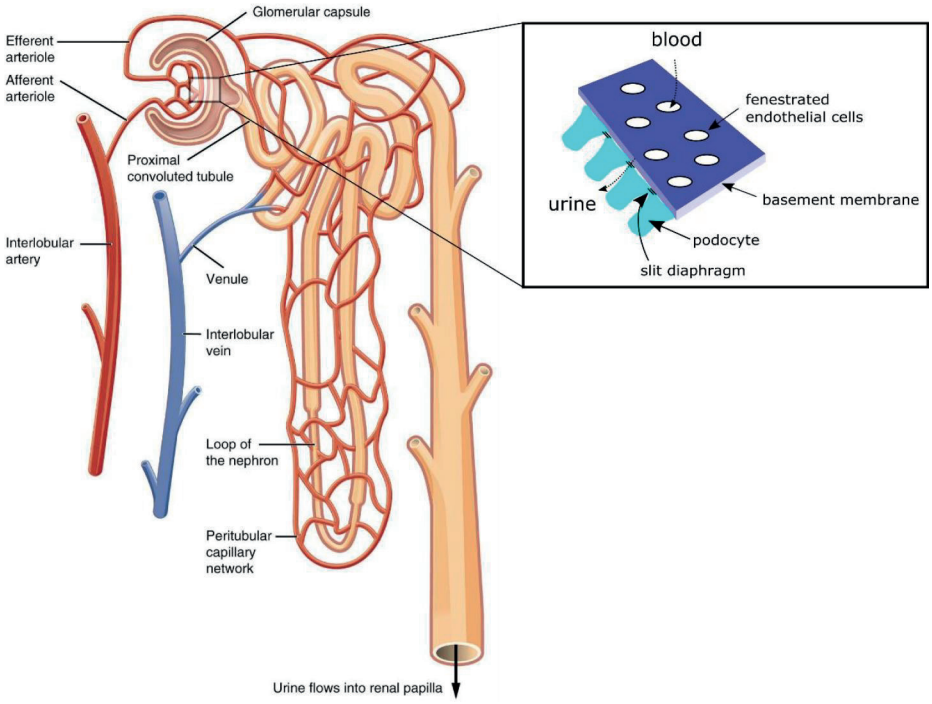

Figure 1. Scheme of the functional unit of the kidney; the nephron (to the left) and the filtration barrier (to the right). Primary urine is filtered from the blood stream in the glomerulus through the fenestrated endothelial cells and the slit diaphragm of the podocytes. The arrows indicate the direction of urine flow. Figure modified and reprinted with permission from Mäkinen 2010 [28].

Oxidative stress is thought to result in endothelial dysfunction, which precedes albuminuria in patients with T1D [29]. Accordingly, glomerular endothelial dysfunction as well as insulin resistance have been suggested to initiate the cascade that results in diabetic nephropathy [30,31].

As DN progresses and the function of the glomerular filtration barrier deteriorates, proteins such as albumin can no longer be withheld in the circulation, but leak into the urine. This is seen as an increase in the albumin excretion rate (AER) from a normal range through microalbuminuria to macroalbuminuria (Table 1, Figure 2).

The increased intra-glomerular pressure caused by the constriction of the efferent glomerular arterioles is thought to result from an activation of the renin-angiotensin system (RAS), a major regulator of blood pressure control. The RAS mediator angiotensin II, which is formed from angiotensin I by the converting enzyme (ACE), stimulates the production of transforming growth factor-β (TGF-β). TGF-β in turn may cause extracellular matrix overproduction [30,32] and induce harmful reactive oxygen species in the kidneys [30,33]. Hyperglycemia induces renal angiotensin II production [34], and medication that blocks the RAS system is used in the treatment of diabetic nephropathy [35]. The activation of fibrotic factors such as TGF-β, connective tissue growth factor, growth hormone, and vascular endothelial growth factor (VEGF) contributes to the formation of fibrotic changes in the glomerulus and the interstitium (Figure 3) [30].

High blood pressure and fibrosis of the kidney affect the glomerular filtration rate (estimated glomerular filtration rate, eGFR), which decreases as diabetic nephropathy progresses and fibrosis of the kidney tissue becomes more evident [14]. When the capacity of the kidney to filter blood is decreased, chronic kidney disease (CKD) commences (GFR <60 ml/min), and when the patient requires dialysis or kidney transplantation for survival, the stage of ESRD is reached. As changes in albumin excretion often precede changes in GFR, AER is commonly used as a clinical marker of DN [36].

Table 1. Kidney status and function defined by the albumin excretion rate (AER), the estimated glomerular filtration rate (eGFR), or the need for dialysis or renal transplant.

	AER limits			**eGFR limits**
normal	<20 μg/min or <30 mg/24 h		**normal**	>90 ml/min
microalbuminuria	≥20 and <200 μg/min or ≥30 and <300 mg/24 h		**reduced**	60–90 ml/min
macroalbuminuria	≥200 μg/min or ≥300 mg/24 h		**chronic kidney disease (CKD)**	<60 ml/min
ESRD	dialysis/transplant		**ESRD**	dialysis/transplant

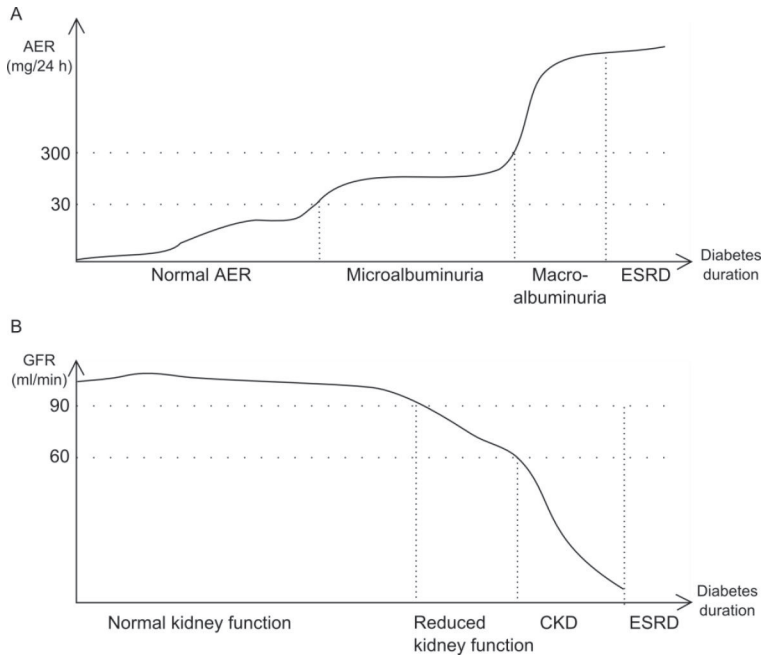

Figure 2. Schematic illustration of the progression of DN. AER, albumin excretion rate; GFR, glomerular filtration rate; CKD, chronic kidney disease.

6.2.2. Biochemistry and molecular cell biology of diabetic complications

Cells use glucose for energy; however, an excess of glucose leads to an overproduction of electron donors to the citric acid cycle. This generates an increased proton gradient over the mitochondrial membrane and leads to the formation of oxygen radicals from the respiratory chain. These mitochondria-derived superoxides cause DNA damage and activate the repair enzyme poly (ADP-ribose) polymerase (PARP). PARP in turn modifies the function of the enzyme glyceraldehyde phosphate dehydrogenase (GAPDH), which is vital for glycolysis. GAPDH inhibition leads to the accumulation of the upstream glycolytic metabolites glyceraldehyde 3-phosphate, fructose 6-phosphate, and glucose, each of which will enter pathways of glucose over-utilization (Figure 3) [37].

A unifying hypothesis has been put forward to explain how hyperglycemia can cause microvascular disease. It suggests that hyperglycemia-induced increases in (1) the polyol pathway flux, (2) the hexosamine pathway flux, (3) the activation of protein kinase C (PKC) isoforms, and (4) increased advanced glycation end product (AGE) formation lead to inflammatory and fibrotic changes and eventually to microvascular diabetic complications (Figure 3) [37,38].

In the polyol pathway, glucose is reduced to sorbitol by the enzyme aldose reductase, a reaction that consumes nicotinamide adenine dinucleotide phosphate (NADPH). Because of this, less NADPH is available to regenerate reduced glutathione (GSH), an antioxidative enzyme, which could induce or exacerbate intracellular oxidative stress [37,38].

The shunting of excess glucose in the form of fructose 6-phosphate to the hexosamine pathway also has potential detrimental effects. This is thought to happen when fructose 6-phosphate is converted to glucosamine 6-phosphate, which can be used for the formation of O-linked glycoproteins. This can modulate transcription factor activity, up-regulate fibrotic protein transcription such as TGF-β and plasminogen activator inhibitor (PAI-1), an inhibitor of fibrinolysis, and down-regulate the function of the vasodilator endothelial nitric oxide (eNOS) [37,38].

Phosphokinase Cs are activated by the lipid second messenger diacylglycerol (DAG), and an increase in glyceraldehyde 3-phosphate leads to the synthesis of DAG. However, both AGEs and elevated reactive oxygen species (such as those produced by the polyol pathway) can further increase DAG. PKC/DAG is involved in processes causing vascular permeability and occlusion, the up-regulation of fibrotic factors such as TGF-β, as well as the activation of pro-inflammatory genes [37,38].

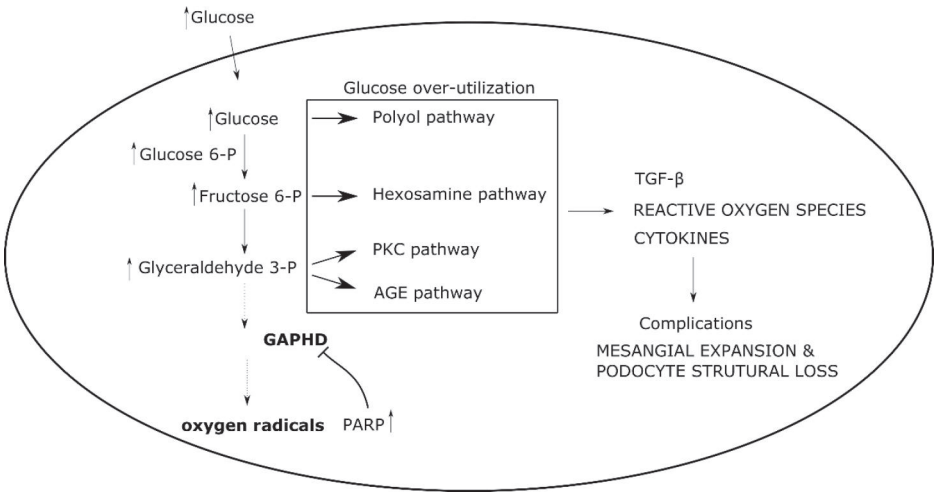

Figure 3. The effect of high intracellular glucose on pathways associated with the development of diabetic complications, adapted from Brownlee 2005 and Satchell 2008 [30,38]. High intracellular glucose causes an excess of electron donors causing oxygen radical formation in the citric acid cycle. Oxygen radicals consequently activate an enzyme involved in DNA repair: the poly (ADP-ribose) polymerase (PARP). PARP inhibits GAPDH activity, leading to an accumulation of upstream glycolytic products, which enter pathways of glucose over-utilization. The glucose over-utilization pathways activate inflammatory and fibrotic pathways causing vascular damage, eventually leading to microvascular complications.

The intracellular formation of reactive dicarbonyl compounds is driven by the excess of glucose and glyceraldehyde 3-phosphate in the AGE pathway. These AGE precursors non-enzymatically react with amino groups on intracellular and extracellular proteins and form AGEs. AGEs can alter the function of intracellular and extracellular proteins and integrins, whereby they become ligands to the receptor for AGE (RAGE) and initiate downstream activation of the inflammatory NF-kB pathway, which in turn can cause vascular pathology [37,38].

6.2.3. Metabolic memory

The importance of good blood glucose control in the prevention of diabetic complications became evident from the Diabetes Control and Complications Trial (DCCT, [39]) and its follow-up study the Epidemiology of Diabetes Interventions and Complications (EDIC, [4]). Intensive insulin treatment in patients with T1D for 6.5 years was associated with a reduced incidence of retinopathy, nephropathy, and neuropathy. This effect remained after the intervention period, although the HbA_{1C} levels between the conventional group and the intensively treated group were no longer different. The term metabolic memory has been used to describe this effect of prolonged protection resulting from early intensive treatment.

In patients with type 2 diabetes, similar beneficial effects of early intensive treatment were reported in the UK Prospective Diabetes Study (UKPDS) and Steno-2 trial [40,41]. On the flip side of the coin, metabolic memory also works in the adverse direction, as patients who later started intensive therapy did worse than those who were exposed to early intensive treatment, even when long-term glucose control was no different between the groups.

Biochemical explanations for metabolic memory include the formation of irreversible AGEs and epigenetic modifications of pro-inflammatory genes such as NFκB that lead to the sustained activation of inflammatory genes [42,43].

6.2.4. The metabolic syndrome and inflammation

In 1988 Reaven proposed the term "syndrome X" to describe the phenomenon in which individuals displaying a cluster of insulin resistance, dyslipidemia, and hypertension were at significantly increased risk of cardiovascular disease [44]. Later, the World Health Organization (WHO) defined the criteria for insulin resistance syndrome and introduced the name metabolic syndrome. Several sets of definitions emphasizing different main aspects have been put forward, until in 2009 a harmonizing consensus statement was issued by the International Diabetes Federation, the National Heart, Lung, and Blood Institute, the American Heart Association, the World Heart Federation, the International Atherosclerosis Society, and the International Association for the Study of Obesity (Table 10, material and methods) [45]. In 2010 WHO reported that rather than being a clinical diagnosis, the metabolic syndrome is a pre-morbid state, and that the concept of the metabolic syndrome is to focus educational attention to this complex of health problems [44].

Central obesity, hypertension, high blood glucose, and dyslipidemia have been associated not only with the progression of DN, but also with cardiovascular morbidity in patients with T1D [46]. With the worldwide increase in obesity, the metabolic syndrome has become a frequently observed condition. In Finnish middle-aged subjects in 2004, 39% of men and 22% of women fulfilled the criteria for the metabolic syndrome [47].

Characteristic of the metabolic syndrome and obesity is insulin resistance. The etiology of insulin resistance is not fully understood; however, chronic tissue inflammation is at least one important causal factor. Cytokines have an adverse effect on intracellular insulin signaling, but also abnormal lipid metabolism can be a mediator of this condition [48]. Often these processes are intertwined and amplify each other; saturated fatty acids can activate the transcription of pro-inflammatory genes that disrupt insulin signaling, and cytokines can increase very low density lipoprotein (VLDL) production in the liver and decrease the function of lipoprotein lipase, leading to a reduced clearance of triglycerides from the circulation. Interestingly, LPS from gram-negative bacteria are also implicated in fat absorption and inflammation [48].

As discussed above, inflammation and insulin resistance are tightly linked with each other, and both have profound effects on the vasculature [14]. At physiological levels, insulin exerts protective effects on the vasculature by decreasing the stiffness of large arteries and inducing the vasodilation of peripheral resistance vessels. This is mediated at least in part by the production of the vasodilator nitric oxide by the endothelium [14]. Impaired endothelial function is one of the earliest findings in the pathogenesis of atherosclerosis and is tightly linked to insulin resistance [49]. Notably, in patients with uncomplicated T1D, the augmentation index (AIx), a proxy for arterial stiffness, is elevated compared to healthy age-matched non-diabetic controls, possibly indicating forthcoming vascular complication [50].

Chronic low grade inflammation is already evident in patients with T1D without complications. Elevated concentrations of CRP, interleukin-6 (IL-6), and fibrinogen have been reported in patients newly diagnosed with T1D [51]. Furthermore, DN is characterized by a chronic low grade inflammation seen as elevated CRP and IL-6 concentrations [8]. Unfortunately, it comes as no surprise that the metabolic syndrome is also a common finding in patients with T1D [46,52].

6.3. Endotoxins

In 1956 Frederick Bang was studying the blood circulation of the horseshoe crab, when one of the crabs died as a result of a *Vibrio* bacterial infection. He discovered that the blood of the crab was entirely jellified, and later found that gram-negative bacterial infections/LPS led to the clotting of the horseshoe crab's blood as part of its defense mechanism [53]. A decade later in the 1960s, Bang and his colleague Levin developed the first endotoxin assay based on this clotting reaction [54]. The horseshoe crab ancestors and their blood-clotting system date back hundreds of millions of years, and still today the detection of endotoxins relies on this creature in the limulus amebocyte lysate (LAL) assay. Nowadays, however, colorimetric readouts are more common than the detection of turbidity/sample clotting.

6.3.1. Structure of LPS

LPS are molecules found at the outer membrane of gram-negative bacteria, contributing to their structural integrity and membrane protection [55]. The molecule consists of three main parts: an O-antigen, a core polysaccharide, and a lipid A part (Figure 4) [56]. The O-antigen is composed of polysaccharides that protrude from the bacterial cell wall, and its structure and composition varies from strain to strain. The core polysaccharide links the O-antigen to the lipid A, and it also contains non-carbohydrate modifications such as phosphates (Figure 4). The presence or absence of the O-antigen determines whether LPS is considered smooth or rough. Full-length O-antigens render the LPS smooth, whereas the absence or reduction of the O-antigen renders the LPS rough, and these modifications have effects on the bacterial function [57].

The toxicity of the LPS molecule arises mainly from the lipid A part, which anchors LPS to the bacterial cell wall. Modifications such as changes in the number of fatty acid chains, chain length, and the level of saturation in the lipid A are associated with its bioactivity [58]. Moreover, bacteria may contain more than one LPS structure, and the numbers and types can be altered within one bacterial population by environmental conditions such as temperature [58]. Bacterial fragments in the circulation containing lipid A activate the immune response, causing fever and, in the worst case, organ failure, as evident during bacterial sepsis [56]. Interestingly, some LPS such as those from *Rhodobacter capsulatus* have protective antagonistic actions on downstream inflammatory signaling [59].

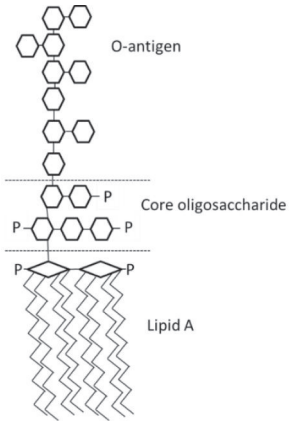

Figure 4. Structure of the LPS molecule of gram-negative bacteria, adapted from [60]. Lipid A mainly conveys the toxicity of the molecule and also integrates it into the cell membrane of the bacteria.

6.3.2. TLR4 receptor

Toll-like receptors (TLR) are central for the innate immune response as they recognize microbial pattern molecules, induce the cytokine inflammatory response, and activate the adaptive immune system [61]. In humans, ten TLRs have been characterized, consisting of TLR1 to TLR10 [62,63]. The function of TLR10 was only recently discovered, and it is the only TLR to have anti-inflammatory effects [64]. TLR3, TLR7, TLR8, and TLR9 localize intracellularly and recognize nucleic acids derived from bacteria and viruses, whereas the rest participate in the pattern recognition at the cell surface of danger signals such as bacterial components (Figure 5) [62,63]. The surface TLRs are activated both by intrinsic factors as well as extrinsic signals such as bacterial components, as shown in Figure 5 [62,63,65].

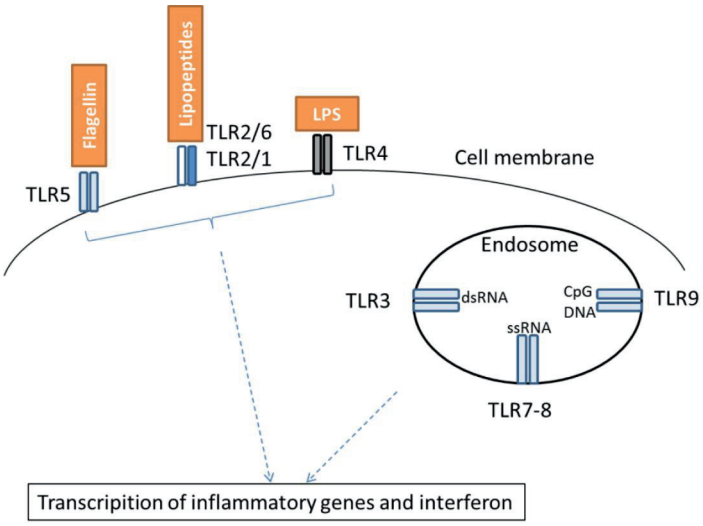

Figure 5. Toll-like receptors (TLRs) in mammals. In humans TLR1–10 exist, out of which TLR3 and TLR7–9 are intracellular. LPS is recognized by TLR4 at the surface of cells. Downstream of TLRs the transcription of pro-inflammatory genes or interferon production is initiated. dsRNA, double-stranded RNA; ssRNA, single-stranded RNA. Picture modified from O'Neill 2013 [63].

LPS are ligands for toll-like receptor 4 (TLR4). In the circulation, LPS is mainly bound by LPS binding protein (LBP), which can transfer it to membrane-bound cluster of differentiation 14 (mCD14) [66]. Membrane-bound CD14 then transfers LPS to a complex consisting of myeloid differentiation 2 (MD-2) and TLR4, leading to signal transduction (Figure 6). LPS can also bind soluble CD14 (sCD14), thereby activating cells that express MD-2 and TLR4 but not mCD14 [66]. Downstream of TLR4, pro-inflammatory cytokines and chemokines are produced, leading to adaptive immune cell recruitment and activation [61].

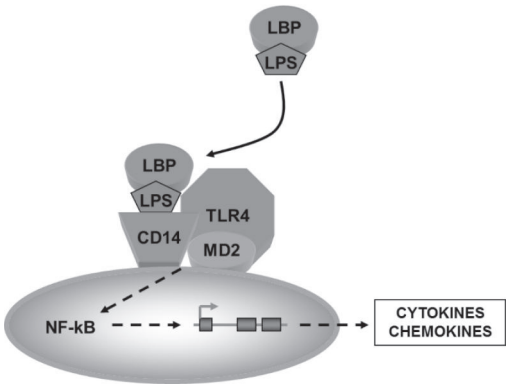

Figure 6. LPS binding to a complex consisting of the TLR 4 receptor, MD-2, CD14, and LBP. Downstream of the receptor complex NF-kB, activation initiates the transcription of pro-inflammatory molecules [61,66].

Other TLR4 ligands involved in diabetes and diabetic nephropathy include heat shock protein (HSP) 60 and HSP70, high mobility group box protein 1 (HMGB1), calprotectin, saturated fatty acids, low density lipoprotein (LDL), and oxidized LDL [67].

In the human kidney, TLR4 is expressed in podocytes, tubular epithelial cells, and mesangial cells [67-69]. Experiments have shown that animals lacking TLR4 (knock-out) are protected from LPS-induced inflammation as well as LPS-induced albuminuria and kidney injury [70,71].

In microalbuminuric and macroalbuminuric patients with type 2 diabetes, transcriptional and translational up-regulation of the TLR4 was described in the glomeruli and tubulointerstitium, which was accompanied by monocyte/macrophage infiltration. Notably, this was specific for diabetes, but not proteinuria, since proteinuric non-diabetic subjects showed no TLR4 activation [71,72]. Moreover, in patients with type 2 diabetes, a high glomerular TLR4 expression was associated with the loss of kidney function six years later [72].

6.3.3. LPS detoxification

Endotoxin detoxification mechanisms can be grouped into (1) molecules that bind LPS and prevent it from interacting with TLR4, (2) enzymes that degrade some part of the LPS molecule, inhibiting its inflammatory capacity, and (3) inactivation by uptake by the liver [66].

Molecules that bind LPS and prevent the interaction with TLR4 include varying classes of lipoproteins (high-density lipoprotein, HDL; LDL; VLDL). Other circulating proteins that bind LPS and inhibit its inflammatory potential include the enzymes intestinal alkaline phosphatase (IAP) by phosphate cleavage, acyloxyacyl hydrolase (AOAH) by lipid A modifications, as well as the cationic antibiotic polymyxin B, which binds to lipid A (Figure 7) [74-77]. In the circulation, LPS is mainly bound to LBP [66]. Moreover, *in vitro* studies have evidenced that a low dose LPS pretreatment lowers the inflammatory responsiveness to secondary LPS exposures. Further, the environment can modulate the response either in a protective way by increasing or maintaining LPS-induced IL-10 production with a low epinephrine dose, or in a harmful way by increasing the vigor of LPS priming with interferon-γ [66]. The number of other putative factors that bind LPS is growing, and some factors are listed in Table 2. Furthermore, in rodents and rabbits, the liver can rapidly remove

LPS from the circulation by the direct binding of LPS to Kupffer cells and transfer to hepatocytes [66].

HDL has anti-atherogenic properties that are primarily attributed to its key role in reverse cholesterol transport. Other effects include improved endothelial cell function, decreased inflammation, and the inhibition of LDL cholesterol oxidation [78]. In conditions of increased oxidative stress, such as diabetes, the formation of oxidized LDL predisposes to cardiovascular disease (reviewed by [73]). Less well recognized is the role HDL plays in innate immunity [78].

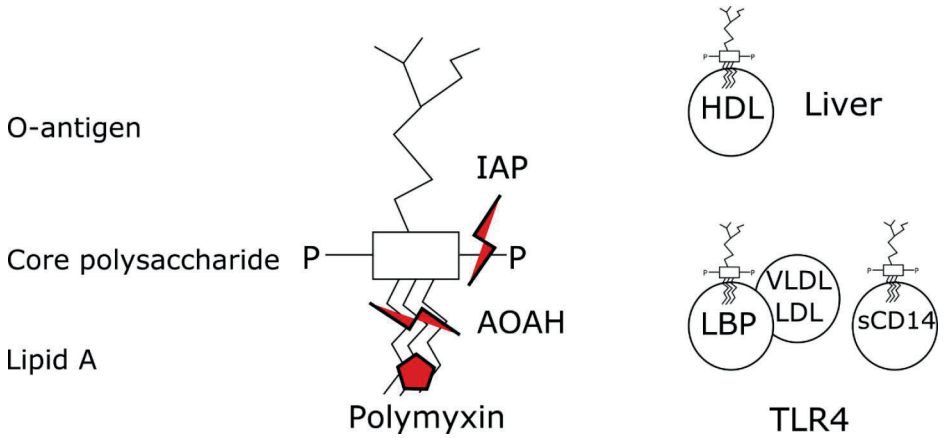

Figure 7. Factors associated with the detoxification of LPS in the circulation. Intestinal alkaline phosphatase (IAP) can cleave phosphate residues from the LPS core polysaccharide, acyloxyacyl hydrolase (AOAH) is a lipase that cleaves fatty acyl chains from lipid A, and polymyxin is an antibiotic that can bind to negatively charged lipid A by its cationic action. Traditional factors in the circulation that bind LPS are HDL particles as well as LPS-binding protein (LBP) and soluble CD14 that transfer LPS to toll-like receptor 4 (TLR4). In conditions of low HDL cholesterol, LPB can redistribute LPS towards the VLDL and LDL particles.

When LPS from gram-negative bacteria or lipoteichoic acid (LTA) from gram-positive bacteria is incubated with whole blood from healthy humans, the majority of these compounds are bound to HDL, which inhibits the interaction with TLRs, dampening macrophage activation. Humans with low HDL concentrations show stronger inflammatory response to LPS administration, and the effect is blunted upon administration of reconstituted HDL. Furthermore, in patients admitted to intensive care, low concentrations of HDL and apolipoprotein A-I (apoA-I) are associated with increased mortality [78].

Both the lipids and proteins that comprise HDL contribute to the neutralization of LPS. ApoA-I alone can neutralize LPS, and the structural modification of apoA-I can alter this interaction. However, lipid emulsions devoid of protein can also neutralize LPS, an effect driven by phospholipids [78]. Finally, the hepatocytes bind LPS and clear them from the circulation by excretion into the bile [86] (Figure 7).

Table 2. A few examples of putative molecules associated with LPS detoxification.

LPS binding molecule	mechanism	evidence	reference
Bactericidal permeability-increasing protein (BPI)[1]	binding of lipid A, bactericidal	*in vitro* studies, clinical associations	[79,80]
Lactoferrin[1]	binding of lipid A	*in vitro* studies, clinical treatment of sepsis in pre-term infants	[81,82]
β defensin 3 (hBD-3) [2]	inhibition of LPS activity, bactericidal	*in vitro* studies, clinical associations	[83]
Cathelicidin (LL-37) [1,3]	inhibition of LPS activity, bactericidal	*in vitro* studies, clinical associations	[83]
Cysteine-rich secretory protein LCCL domain containing 2 (CRISPLD2)[4]	binding of lipid A	*in vitro* studies with human leukocytes, *in vivo* studies in rodents, human septic shock	[84,85]

Main sources: [1]neutrophils, [2]gingival crevicular fluid, oral tissue, [3]epithelial cells, [4]white blood cells, and multiple organs.

6.3.4. Other lipoproteins and LPS

Since LPS is a lipid soluble molecule, it can be transported together with other lipoproteins, apart from HDL, in the circulation. Especially in conditions where HDL concentrations may be low, such as atherosclerosis, periodontitis, and bacterial sepsis, the role of the LBP is pronounced as it has a high affinity for the apoB-containing lipoproteins VLDL and LDL, leading to LPS scavenging [87-89]. Importantly, the association of LPS to VLDL and intermediate density lipoprotein (IDL) fractions in patients with periodontal disease associated positively, and LPS in the LDL and HDL fractions negatively, with clinical periodontal parameters and plasma cytokine concentrations [89].

Chylomicrons transport dietary fats from the intestine into the circulation and can be distinguished from other lipoproteins by the presence of apoB-48. The master regulator of chylomicron turnover is apoE, which facilitates chylomicron remnant binding to the specific hepatic receptor low density lipoprotein-receptor related protein 1 (LRP1) [90]. Electron microscopy images have shown that LPS from the gut is found in conjunction with chylomicrons [91], highlighting the importance of apoB-48 and apoE in LPS metabolism.

6.3.5. Sources of LPS

Both infections and commensal bacteria are potential sources of systemic LPS. The gut contains a large pool of bacteria and thus serves as a reservoir of endotoxins/LPS. Animal experiments led to the hypothesis that the lipid soluble endotoxins from the gut gain access to the circulation by active transport when packed into chylomicrons [92] or by passive leakage through the gut epithelia [93]. In animal experiments after high-fat feeding, a twofold to threefold increase in circulating endotoxin activity was observed, which was defined as metabolic endotoxemia [94]. Also in humans a positive association between

energy intake and serum LPS activity in healthy men has been reported based on food diaries [95]. A few human studies have shown a transient increase in circulating endotoxins following the consumption of energy-rich meals [91,96-98]. Moreover, food-derived LPS [99] as well as saliva LPS (unpublished observation, Lassenius) can possibly contribute to the endotoxin load in the intestine, even though their contribution is probably minor compared to the amount of intestinal gram-negative bacteria. However, many studies show no association between high-fat meals and an increase in circulating inflammatory factors such as CRP, IL6, and serum amyloid A (SAA) [100]. This may depend on differences in study design, fat quality, and blood sample timing, as well as slow responses in inflammatory proteins.

LPS molecules are postulated to contribute to the initiation of low grade inflammation and metabolic alterations, such as insulin resistance [93,94]. Cross-sectional studies have shown that endotoxin correlates with markers and conditions of insulin resistance, with endotoxin acting as a predictive metabolic biomarker of type 2 diabetes [7,101,102]. Intravenous LPS administration (3ng/kg) in healthy adults was shown to result in insulin resistance and changes in molecules associated with insulin receptor signaling in adipose tissue [103]. The relationship of LPS activity (EU/ml) to LPS concentration (ng/ml) is not easily established, since different sizes of LPS molecules have varying activity. However, increases in LPS activity are evident in metabolic endotoxemia and sepsis in mammals (Table 3).

Table 3. LPS activity in sepsis and metabolic endotoxemia in mammals.

	LPS activity (EU/ml)
Sepsis	> 3-fold increase [104]*
Metabolic endotoxemia	2-fold increase [94]**

*Humans were compared to healthy controls. **Rodents with 2 weeks of high-fat diet were compared to mice on normal chow.

However, the limitation of many studies investigating the consequences of postprandial endotoxemia in humans is the inability to accurately distinguish between an increase in postprandial endotoxemia and an increase in serum lipemia [96-98], since lipemia/sample turbidity may give false positive results in the LAL assay [105]. Only one study reports the use of a kinetic measurement to reduce background absorption from lipemia in the LPS measurement [91].

Apart from the gut, other possible entrance routes for LPS include the oral cavity. In periodontitis pathogens and their virulence, factors such as LPS have access to the circulation via inflamed periodontal tissue. These patients suffer from bacteremia and endotoxemia [106,107]. Epidemiological data suggest a close association between periodontal health and chronic kidney disease, possibly driven by inflammation [108]. Moreover, periodontitis is also associated with cardiovascular disease [109]. Interventions to improve periodontal disease have been associated with improvements in kidney function [110], implying a true causative relationship between oral health and kidney function.

6.4. The gut microbiome and type 1 diabetes

Accumulating evidence suggests that the gut microbiome may protect against some metabolic diseases, such as T1D, by promoting intestinal homeostasis. In a small study with four children who developed T1D, preceding diagnosis, the microbiome was found to be

less diverse and stable than in matched controls, indicating that the altered microbiome may predispose to disease onset [111].

Animal studies in non-obese diabetic (NOD) mice have shown the contribution of increased gut permeability to the regulation of autoimmunity. *Citrobacterium rodentum* infection causing intestinal barrier disruption accelerated the development of autoimmune diabetes. Treatment with modified bacteria lacking the virulence factor causing barrier disruption caused no aggravation of diabetes. The bacteria were administered to the stomach, after which wild type *C. rodentum* was found in the pancreatic lymph nodes, suggesting that gut barrier disruption may have led to β-cell specific autoimmunity and contributed to β-cell destruction [112]. Several studies have shown increased gut permeability using a lactulose-mannitol test in patients with T1D [113-116]. The composition of intestinal microbiota affects gut permeability, but gut immune-modulating effects also associate with oral tolerance to extrinsic antigens that may be involved in the development of T1D [112].

6.4.1. Short-chain fatty acids

Short-chain fatty acids (SCFA; proprionate, butyrate, acetate, valerate) are produced by intestinal microbiota as they process non-digestible dietary carbohydrates (reviewed by [117]). These molecules have profound effects on the host; in addition to serving as an energy source for enterocytes, SCFAs regulate gene expression by modulating histone deacetylases, and their receptors are present in many tissues such as adipose tissue, the spleen, bone marrow, and peripheral blood mononuclear cells (reviewed by [117]). Butyrate has anti-inflammatory properties on the intestinal lining, an effect mediated in part by IAP expression [74]. Conditions of intestinal inflammation such as IBD are associated with lower fecal SCFA levels as well as a reduced number of butyrate-producing bacteria [118,119].

6.4.2. Fecal immunoglobulins

The host controls the fecal microbiota and the uptake of molecules into the circulation by secreting immunoglobulins into the intestine (reviewed by [120]). The most prevalent immunoglobulins in the intestine are secretory immunoglobulin A (IgA). To a lesser extent, also IgG and IgM molecules are present. These molecules protect the host by immune exclusion, which prevents the interaction of antigens with the mucosal surface, but also affects the composition of the microbiota (reviewed by [120]).

Conditions of intestinal inflammation, such as IBD, alters fecal immunoglobulin levels to microbial and food antigens [121]. Dietary oxidized lipids strongly affect lipoprotein oxidization in the circulation and associate with foam cell formation and the initiation of vascular disease (reviewed by [122,123]). Hence, secretory IgA antibodies in the intestine can affect disease-associated parameters such as circulating oxidized LDL levels.

6.5. Mechanisms of endotoxemia-induced kidney injury

Cytoskeletal remodeling is essential for podocytes, and proteinuric kidney diseases are typically driven by the rearrangement of the podocyte microfilament system, causing foot process effacement and slit diaphragm disruption [124]. In animal models, LPS is frequently used to induce nephrosis and proteinuria.

Mice lacking cathepsin L, B7-1, and urokinase plasminogen activator receptor (uPAR) are resistant to LPS-induced proteinuria [9-11]. Downstream of TLR4, the co-stimulatory protein B7-1 (also called CD80) is up-regulated in mouse podocytes in response to an LPS

injection. B7-1 is involved in the activation of T-cells by antigen-presenting cells, and its activation was shown to cause cytoskeletal actin remodeling and foot-process effacement associated with proteinuria [9]. An increase in the cytosolic protease cathepsin L has been shown in the podocytes and tubular cells of patients with diabetic nephropathy. In mice, LPS injection causes a similar up-regulation of this protease, leading to cleavage of dynamin, a protein normally involved in actin dynamics, and causing cytoskeletal rearrangements associated with proteinuria [11].

The uPAR is a glycosylphosphatidylinositol (GPI)-anchored protein. It has been shown to be a proteinase receptor for urokinase, but has also been involved in nonproteolytic pathways, mainly through its ability to form signaling complexes with other transmembrane proteins such as integrins, caveolin, and G-protein-coupled receptors. uPAR is also up-regulated in the glomeruli of patients with diabetic nephropathy and in animal models in response to LPS [10]. Animal models have shown that uPAR is required for LPS-induced foot-process effacement via the activation of αvβ3 integrin and its effect on podocyte motility [10]. How these pathways cooperate in the development of LPS-induced proteinuria remains an open question.

Recently, 3-phosphoinositide-dependent kinase-1 (PDK-1), a protein involved in cell survival, was shown to be down-regulated in the glomeruli of patients with type 2 diabetes and diabetic rats prior to proteinuria. LPS treatment or treatment with patient sera high in LPS had the same down-regulatory effect on PDK-1 in the podocytes and led to an increase in apoptosis. PDK-1 down-regulation and apoptosis was prevented by TLR4 receptor blockade by GIT-27 [125]. Taken together, there is strong evidence that LPS is a potent inducer of kidney injury and is associated with increased glomerular permeability.

7. Aims

The main aims of this thesis were the following:

I. To investigate whether bacterial endotoxins contribute to the development or progression of diabetic nephropathy.

II. To explore the association of endotoxins and components of the metabolic syndrome both in patients with type 1 diabetes and non-diabetic controls.

III. To study the effects of multiple high-fat meals on circulating endotoxins, inflammation, vascular function, and lipid metabolism in patients with type 1 diabetes and non-diabetic controls.

IV. To study the role of alkaline phosphatase in gut homeostasis and inflammation in patients with type 1 diabetes with and without diabetic nephropathy as well as in non-diabetic controls.

8. Material and Methods

8.1. The Finnish Diabetic Nephropathy Study (FinnDiane)

All individuals studied in this thesis were recruited by the FinnDiane Study. The FinnDiane Study was initiated in 1997 with the aim to investigate T1D and environmental as well as genetic factors affecting the development of diabetic complications. A special emphasis is put on understanding the etiology of diabetic nephropathy. The FinnDiane Study is a Finnish nationwide multi-center study, and about 5000 participants have been investigated at the different study centers (Figure 8). This represents about 12.5% of all patients with T1D in Finland. When FinnDiane was initiated, all adults with T1D were invited to take part in the study, and the participation rate was 78% [126].

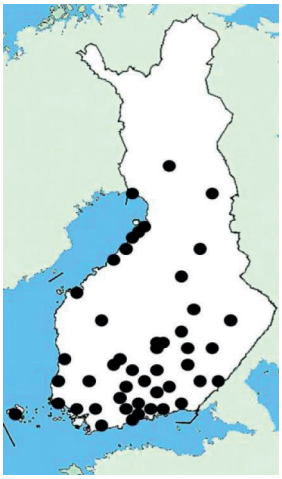

Figure 8. The FinnDiane centers in Finland.

8.2. Ethical aspects

The FinnDiane Study protocol was approved by the Ethics Committee of Helsinki University Hospital (decision number 491/E5/2006) as well as by the local ethics committees at the local study centers and is being conducted in accordance with the Helsinki declaration. The high-fat diet study was also approved by the Ethics Committee of Helsinki University Hospital (decision number 221/13/03/01/2009). Written informed consent is given by all patients prior to participation in the study. All data used for analyses are coded with ID numbers, and personal information is only known to the FinnDiane researchers.

Because of the observational nature of the FinnDiane Study, no interventions are carried out. The only potential inconvenience to patients is the possible pain caused by venipuncture when blood samples are drawn, as well as the extra time spent at study visits and in completing questionnaires.

8.3. Subject recruitment

All subjects with T1D were enrolled into the FinnDiane Study by nurses and doctors at the local study centers for Studies I and II. Individuals with T1D were defined as <40 years of age at onset and insulin treatment initiated within one year of diagnosis. In Study I, progressors and non-progressors were matched for age and sex. The baseline clinical characteristics for the participants in Study I are presented in Table 4.

Overweight subjects in Study II were selected from two population-based prospective studies, FinnTwin16 (FT16) and FinnTwin12 (FT12), each consisting of five consecutive birth cohorts of Finnish twins. One twin from each pair was randomly selected, and the group was split into lean non-diabetic controls (NDC_lean) and overweight non-diabetic controls (NDC_ow) by a BMI cut off of 25 kg/m^2. In the same study, patients with IgA nephropathy were investigated at the Department of Internal Medicine at Tampere University Hospital (Tampere, Finland). IgA nephropathy (IgAGN) was defined as glomerulonephritis with IgA as the sole or main glomerular immunofluorescence finding in a renal biopsy specimen. The baseline clinical characteristics of the participants in Study II are presented in Table 5 for patients with T1D, and Table 6 for non-diabetic study subjects.

Table 4. Baseline clinical characteristics of the patients with type 1 diabetes and normal AER or macroalbuminuria at baseline in Study I.

	Normal AER	**Macroalbuminuria**	***P*-value**
N (M/F)	239 (150 / 89)	238 (149 / 89)	
Age (years)	32 ± 11	41 ± 10	<0.001
Age at onset (years)	16 ± 9	11 ± 7	<0.001
Duration (years)	17 ± 11	29 ± 8	<0.001
Follow-up (years)	6.2 (4.4-7.2)	6.8 (5.7-7.4)	0.001
HbA_{1c} (%)	8.5 ± 1.6	8.9 ± 1.5	0.004
BMI (kg/m^2)	24.7 ± 3.3	25.8 ± 4.1	0.001
Waist-to-hip ratio	0.86 ± 0.08	0.90 ± 0.09	<0.001
Diastolic BP (mmHg)	128 ± 15	144 ± 20	<0.001
Systolic BP (mmHg)	79 ± 9	83 ± 10	<0.001
Triglycerides (mmol/l)	1.07 (0.80-1.44)	1.42 (1.03-2.09)	<0.001
Cholesterol (mmol/l)	4.8 ± 1.0	5.4 ± 1.1	<0.001
HDL cholesterol (mmol/l)	1.33 ± 0.37	1.17 ± 0.38	<0.001
LDL cholesterol (mmol/l)	2.90 ± 0.85	3.44 ± 0.93	<0.001
ApoA-1 (mg/dl)	138 ± 20	140 ± 24	ns
ApoA-2 (mg/dl)	35 ± 8	34 ± 7	ns
ApoB-100 (mg/dl)	88 ± 20	103 ± 23	<0.001
CRP (mg/l)	1.9 (1.2-3.6)	2.7 (1.6-5.4)	<0.001
eGFR (ml/min/1.73m^2)	96 (83-107)	53 (29-74)	<0.001
AER (mg/24h)	10 (7-17)	626 (225-1497)	<0.001
eGDR (mg/kg^{-1}·min^{-1})	7.4 (5.7-9,0)	4.1 (3.1-5.0)	<0.001
AHT medication (%)	10	92	<0.001
Lipid medication (%)	4	25	<0.001
Current smoking (%)	32	32	ns

Data are expressed as mean ± SD or median (25^{th}–75^{th} quartile). AER, albumin excretion rate; eGFR, estimated glomerular filtration rate; eGDR, estimated glucose disposal rate; AHT, anti-hypertensive medication.

Table 5. Clinical characteristics of type 1 diabetic patients in Study II.

	Normal AER	Microalbuminuria	Macroalbuminuria
N (M/F)	587 (257/330)	144 (65/79)	173 (110/63)*
Age (years)	44 (36-53)	46 (37-55)	48 (40-56)†
Age at onset (years)	17 (10-25)	11 (6-19)*	11 (7-16)*
Duration of diabetes (years)	28±12	33±11*	35±9*
HbA_{1C} (%)	7.7±1.3	7.8±1.8	7.8±2.0
BMI (kg/m^2)	25.6±4.2	26.4±4.2†	27.0±4.9*
Systolic BP (mmHg)	136±17	141±19†	146±18*
Diastolic BP (mmHg)	78±9	78±10	80±11†
Triglycerides (mmol/l)	0.90 (0.69-1.21)	0.95 (0.72-1.51)†	1.35 (0.93-2.03)*
Cholesterol (mmol/l)	4.7±0.8	4.9±0.8†	4.8±1.1
HDL cholesterol (mmol/l) M	1.5±0.4	1.4±0.4	1.3±0.4†
HDL cholesterol (mmol/l) F	1.7±0.4	1.8±0.5†	1.5±0.4*
ApoA-1 (mg/dl)	146±32	152±34	143±33
ApoB-100 (mg/dl)	75±19	78±18	85±25*
CRP (mg/l)	1.0 (0.3-2.5)	1.0 (0.5-2.5)	1.1 (0.6-2.9)
eGFR ($ml/min/1.73m^2$)	102 (90-111)	96 (81-108)*	57 (35-84)*
LPS (EU/ml)	57 (50-69)	56 (47-72)	67 (52-96)*
LPS/HDL ratio	37 (29-50)	35 (27-52)	50 (34-77)*
Lipid medication (%)	22	35*	56*
AHT medication (%)	31	79*	98*
Smoking (%)	17	20	26*
Retinopathy (%)	20	55*	78*
Coronary heart disease (%)	6	9	15*

Patients with normal AER are used as a reference group in all comparisons. Data are expressed as mean ± SD or median (25^{th}–75^{th} quartile). †$p<0.05$, *≤ 0.001. eGFR, estimated glomerular filtration rate; AHT, anti-hypertensive medication; LPS, lipopolysaccharide.

Table 6. Clinical characteristics of non-diabetic study subjects in Study II.

	NDC_lean	NDC_ow	NDC_all	IgAGN
N (M/F)	219 (96/123)	126 (81/45)*	345 (177/168)	98 (61/37)
Age (years)	33±10	33±9	33±10	52±13*
BMI (kg/m^2)	22.2±1.7	28.2±2.8*	24.3±3.6	26.5±4.3*
Waist M (cm)	85 (80-88)	97 (92-102)†	89 (84-96)	91 (87-100)
Waist F (cm)	76 (72-81)	90 (86-98)*	79 (74-85)	80 (71-87)
Systolic BP (mmHg)	123±14	130±13*	126±14	148±21*
Diastolic BP (mmHg)	75±8	80±8	77±8	90±11*
Glucose (mmol/l)	4.8 (4.5-5.1)	5.0 (4.7-5.4)*	4.9 (4.6-5.2)	4.7 (4.4-5.1)†
Insulin (IU/ml)	4.2 (2.9-5.5)	5.7 (4.4-10.0)*	4.8 (3.3-6.7)	7.7 (6.1-10.7)*
HOMA-IR	1.0 (0.8-1.5)	1.3 (0.8-2.0)†	1.1 (0.8-1.6)	1.6 (1.1-2.2)*
Triglycerides (mmol/l)	0.8 (0.7-1.1)	1.1 (0.8-1.4)	0.9 (0.7-1.2)	1.2 (0.8-1.6)*
Cholesterol (mmol/l)	4.5±0.9	4.9±0.9*	4.7±0.9	5.3±1.1*
HDL M (mmol/l)	1.5±0.3	1.4±0.3†	1.4±0.3	1.3±0.3†
HDL F (mmol/l)	1.8±0.3	1.6±0.4†	1.7±0.4	1.8±0.4
CRP (mg/l)	0.7 (0.5-1.5)	1.2 (0.7-3.3)*	0.9 (0.5-2.0)	1.7 (0.8-2.9)*
LPS (EU/ml)	60 (44-80)	62 (49-82)	61 (44-79)	49 (40-55)*
LPS/HDL ratio	37 (28-51)	45 (35-62)*	41 (30-54)	34 (26-43)*
Lipid medication (%)	1	0	1	19*
AHT medication (%)	2	4	3	56*
Smoking (%)	14	21	16	15

NDC_lean are compared to NDC_ow and IgAGN to NCD_all. Data are expressed as mean ± SD or median (25th–75th quartile). †$p<0.05$, *≤ 0.001. NDC, nondiabetic control; lean, BMI <25kg/m^2; ow, overweight BMI ≥25kg/m^2; IgAGN, IgA glomerulonephritis. AHT, anti-hypertensive medication; LPS, lipopolysaccharide.

8.3.1. High-fat diet study

The subjects included in Studies III and IV were recruited by the FinnDiane center in Helsinki. A study on the effects of a high-fat diet on endotoxemia, inflammation, vascular function, and lipid metabolism was conducted between the years 2009 and 2013. In Study III patients with T1D and an AER < 200µg/min were included and compared to non-diabetic controls, whereas in Study IV patients with microalbuminuria were excluded. In Study IV comparisons were made between non-diabetic controls and patients with T1D and normal AER on one hand and patients with macroalbuminuria and those with normal AER on the other.

Inclusion criteria for both studies III and IV were age <65 years, no smoking, no use of antibiotics during the past month, and no visits during the past month outside the Nordic countries.

Participants were given three energy-rich meals during the study day (Figure 9): breakfast (8:00, 965 kcal, fat energy 58%), lunch (12:00, 870 kcal, fat energy 44%), and dinner (16:00, 779 kcal, fat energy 46%). Blood samples were drawn at fasting and every two hours until 18:00. Prior to the study day, participants kept a three-day food record. Leukocyte counts were assessed at fasting and at 18:00. Stool samples were collected before and after the study day. The fasting patient characteristics are presented in Table 7 for Study III and Table 8 for Study IV.

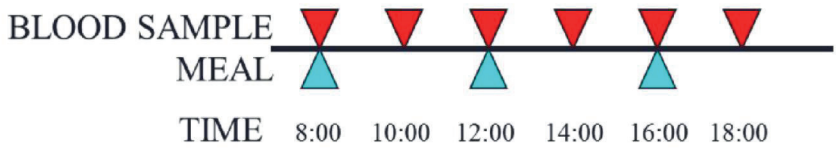

Figure 9. Design of the high-fat diet study.

8.4. Sample storage

The serum for Studies I and II and urine (Study II) samples were kept frozen at -20°C until the measurement of LPS activity and the determination of urinary monocyte chemoattractant protein 1 (MCP-1). For studies III and IV, serum and fecal samples were stored at -80°C until time of measurement. In all studies, serum lipids, CRP, creatinine (serum and urine), and urinary albumin were measured in batches shortly after sample acquisition. Glycated hemoglobin as well as blood glucose was always measured on the same day of sample retrieval.

8.5. Patient characterization

The FinnDiane Study protocol includes the assessment of anthropomorphic measures: height, weight, and waist and hip circumference. BMI was calculated as weight/height^2 (kg/m^2) and waist-to-hip ratio by dividing waist circumference with hip circumference.
Blood pressure was measured twice in the sitting position after a 10-minute rest with an automated blood pressure measuring device; the average of the readings was used for the analyses. In Study III an applanation tonometer was used to assess arterial stiffness (SphygmoCor, Atcor Medical, Sydney, Australia). Body fat percentage was measured by bioimpedance. Smoking and medication were self-reported on a questionnaire during the visit to the FinnDiane center.

8.5.1. Lipids

Serum triglycerides, HDL cholesterol, apoA-I, apoA-II, and apoB-100 were measured with a Konelab analyzer using automated enzymatic methods (Thermo Scientific, Vantaa, Finland) in Professor Marja-Riitta Taskinen's research laboratory at the Helsinki University Central Hospital, Heart and Lung Center, Cardiovascular Research Group. LDL cholesterol was calculated using the Friedewald formula if triglycerides were below 4.0 mmol/l [127].

In addition, for studies III and IV, plasma apoB-48 (AKHB48, Shibayagi Co Ltd, Shibukawa, Japan) was measured in Professor Taskinen's laboratory. ApoE concentrations [128] and the activity of enzymes involved in lipoprotein metabolism, phospholipid transfer protein (PLTP) [129], cholesteryl ester transfer protein (CETP) [130,131] and paraoxonase (PON1) [132], were measured in Professor Matti Jauhiainen's research laboratory at the National Institute of Health and Welfare, Public Genomics Unit, Helsinki, Finland.

Nuclear magnetic resonance (NMR) was used to assess the HDL particle size (Study III) in Professor Mika Ala-Korpela's laboratory at the University of Oulu, Computational Medicine, as previously described [133].

8.5.2. Creatinine

Serum creatinine was measured with a kinetic Jaffé reaction until January 2002. Thereafter, a photometric enzymatic method was applied. The correlation coefficient between the two methods is 0.988. The following conversion formula enabled usage of both measurement methods: S-Creatinine (IDMS) = 0.953 x S-Creatinine Jaffé) – 7.26. Urinary creatinine was measured in HUSLAB.

8.5.3. Glycosylated hemoglobin

Glycosylated hemoglobin (HbA_{1C}) was determined locally at each center by standardized assays. All laboratories are accredited and participate in a national quality assessment on a regular basis. For Study II twenty blood samples were sent to Tampere (TAYS) and Helsinki University Hospital Laboratories (HUSLAB) for the comparison of measurements of HbA_{1C}; the samples had an overall Pearson's correlation of r=0.94, and the sample mean ± SD for TAYS was 39.7±6.3 mmol/mol and for HUSLAB 36.7±7.8 mmol/mol.

8.5.4. Inflammatory markers

Serum high-sensitive CRP was measured by a Hitachi automated analyzer (Orion Diagnostica, Espoo, Finland) until April 21, 2008, after which the concentrations were measured by a kit from Roche (both at HUSLAB). The correlation between the methods was r=0.98. For Studies II, III, and IV, hsCRP was measured in Professor Taskinen's laboratory by an automated analyzer (hsCRP kit 981798; Konelab, Thermo Scientific, Vantaa, Finland). In addition to CRP, specific inflammatory cytokines were measured as described below. Urinary monocyte chemoattractant protein 1 (uMCP1; Study II) concentrations were measured according to the manufacturer's instructions by ELISA (R&D Systems, Abingdon, U.K.) and normalized against urinary creatinine. For the uMCP-1 measurements, the coefficient of variation (CV) of one sample in one plate (intra CV) was 5% and the variation between plates (inter CV) 12%.

For Studies III and IV, interleukin 6 (IL-6 – intra CV 5%, inter CV 9%) and serum amyloid A (SAA – intra CV 2%, inter CV 18%) were measured according to the manufacturer's

instructions by ELISA (Quantikine IL6, Quantikine human SAA; R&D Systems, Abingdon, U.K.).

8.5.5. Food diaries

For studies III and IV, patients kept a three-day food diary prior to the investigation day in which they reported on food intake and insulin administration. The micronutrient and macronutrient intake was calculated using a computer program to dissect the composition of the food (AivoDiet v. 2.0.2.3, Aivo Finland, Turku, Finland).

8.5.6. Matching data

In Study I, those that progressed with regard to kidney disease were matched for age and sex with patients who did not progress during the follow-up time, since both age (or diabetes duration) and sex are associated with adverse renal outcomes. In Study III, patients with T1D and non-diabetic controls were of similar age and sex, even if not individually matched.

In Study II, patients with IgAGN and diabetic kidney disease were matched for sex and eGFR in order to more closely look at LPS activity and renal disease and compare the differences between two etiologically different diseases.

In Study IV, patients with T1D and normal AER had a significant difference in age, six years on average, compared to non-diabetic controls (43±10 vs. 37±11, p=0.011). Patients with or without kidney disease showed no difference in age between the groups. After adjusting for age in the analyses comparing patients with T1D and normal AER and non-diabetic controls, the significance disappeared for fecal calprotectin (adjusted p = 0.063) and fecal total IgA concentrations (adjusted p = 0.068); however, the differences in all other analyses remained. The discrepancy in calprotectin and total IgA significance likely arose because the groups are relatively small (N_{T1D} 36, $N_{control}$ 41). Biologically, however, fecal calprotectin concentrations are relatively stable; only slight variation has been reported in different age groups of healthy subjects: 10–19 years (calprotectin 27±14 μg/g), 20–39 years (24±12 μg/g), 40–59 years (25±13 μg/g) [134].

Table 7. Fasting clinical characteristics of the participants in Study III.

	Non-diabetic controls	T1D	*P*-value
N (M/F)	18/16	16/21	ns
Age (yrs)	38.2 ± 10.3	42.5 ± 9.4	ns
Duration (yrs)	-	26 ± 13	-
HbA_{1c} (%) (mmol/mol)	5.3 ± 0.3 (34 ± 3.3)	8.0 ± 1.3 (64 ± 14.2)	<0.001
BMI (kg/m^2)	25.2 ± 4.2	26.1 ± 3.6	ns
Systolic blood pressure (mmHg)	129 ± 14	133 ± 17	ns
Diastolic blood pressure (mmHg)	77 ± 10	77 ± 8	ns
Blood glucose (mmol/l)	4.9 ± 0.6	8.7 ± 3.4	<0.001
Triglycerides (mmol/l)	1.0 ± 0.3	0.9 ± 0.5	ns
Cholesterol (mmol/l)	4.7 ± 0.7	4.6 ± 0.7	ns
HDL cholesterol (mmol/l)	1.3 (1.1-1.7)	1.6 (1.3-1.8)	ns
LDL cholesterol (mmol/l)	2.8 ± 0.7	2.6 ± 0.7	ns
ApoA-1 (mg/dl)	138 (123-158)	153 (132-162)	ns
ApoB-100 (mg/dl)	83 (73-93)	72 (66-87)	ns
ApoB-48 (mg/dl)	3.8 (2.7-5.4)	5.0 (3.7-8.8)	0.014
CRP (mg/l)	1.0 (0.2-2.7)	1.5 (0.3-5.4)	ns
Interleukin-6 (pg/ml)	1.2 (0.8-2.0)	1.5 (0.5-3.5)	ns
AER (mg/24h)	3.4 (2.4-5.2)	7.0 (3.8-13.4)	0.001
eGFR ($ml/min/1.72m^2$)	102 (93-110)	101 (94-114)	ns
LPS (EU/ml)	0.82 (0.57-1.43)	0.90 (0.52-1.24)	ns

eGFR, estimated glomerular filtration rate; AER, albumin excretion rate; LPS, lipopolysaccharide. Data are expressed as mean ± SD or median (25^{th}–75^{th} quartile). T1D, type 1 diabetes (albumin excretion <200µg/min or <300mg/24h).

Table 8. Fasting clinical characteristics of the participants in Study IV.

	Non-diabetic controls	T1D normal AER	T1D macroalbuminuria
N (M/F)	20/21	14/22	7/3
Age (yrs)	37 ± 11	43 ± 10 †	48 ± 12
Diabetes duration (yrs)	---	26 ± 14	35 ± 12
HbA_{1c} (%)	5.3 ± 0.3	8.0 ± 1.3 **	8.3 ± 1.5
BMI (kg/m^2)	25.5 ± 4.9	26.0 ± 3.9	26.6 ± 5.1
Systolic blood pressure (mmHg)	129 ± 14	131 ± 12	158 ± 21 **
Diastolic blood pressure (mmHg)	78 ± 10	77 ± 8	78 ± 7
Blood glucose (mmol/l)	4.9 ± 0.6	8.7 ± 3.4 **	8.6 ± 2.5
Triglycerides (mmol/l)	0.98 ± 0.33	0.99 ± 0.54	1.20 ± 0.32 †
Cholesterol (mmol/l)	4.7 ± 0.8	4.6 ± 0.8	3.9 ± 0.6 †
HDL cholesterol (mmol/l)	1.38 ± 0.39	1.53 ± 0.34	1.29 ± 0.41
ApoA-1 (mg/dl)	140 ± 24	149 ± 22	138 ± 23
ApoB-100 (mg/dl)	81 ± 20	80 ± 25	72 ± 19
ApoB-48 (mg/dl)	4.2 ± 2.5	7.0 ± 5.4 *	12.7 ± 7.5 *
ApoE (mg/dl)	30 (20-35)	23 (14-37) †	22 (16-33)
CRP (mg/l)	1.2 (0.3-2.9)	1.2 (0.2-4.4)	0.7 (0.0-2.8)
AER (mg/24 h)	3.5 (3.0-5.8)	5.0 (3.3-10.5) *	978 (231-2812) **
eGFR ($ml/min/1.72m^2$)	104 (95-116)	103 (91-115)	69 (42-101) **
LPS (EU/ml)	0.8 (0.6-1.3)	0.9 (0.6-1.3)	0.7 (0.6-0.8)
Serum Total-AP (U/L)	59 (47-71)	57 (45-67)	71 (55-92)
Intestinal-AP (U/L)	0.00 (0.00-1.7)	0.60 (0.00-7.00) †	1.75 (0.00-3.30)
Blood group A (%)	51.3	27.8	60.0
Blood group AB (%)	5.1	8.6	10.0
Blood group B (%)	10.3	25.7	10.0
Blood group O (%)	33.3	37.1	20.0
FUT2 secretory phenotype (%)	79.5	80.0	70.0
Blood pressure medication (%)	12	36 †	90 *
Lipid medication (%)	2	28 *	70 †

Patients with type 1 diabetes (T1D) and normal albumin excretion rate (AER) are compared with non-diabetic controls; patients with macroalbuminuria are compared to patients with normal AER. eGFR, estimated glomerular filtration rate; AP, alkaline phosphatase; LPS, lipopolysaccharide. Data are expressed as mean ± SD or median (25^{th}–75^{th} quartile). †$p<0.05$, *$p\leq0.01$, **$p\leq0.001$.

8.6. Periodontal pathogens

Serum IgA and IgG antibodies to the periodontal pathogens *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* were measured from serum samples by a multiserotype ELISA (Study I) in the laboratory of Docent Pirkko Pussinen at the University of Helsinki, Institute of Dentistry, as previously described [135].

8.7. Kidney status and function

Kidney status was based on albumin excretion rate (AER) from at least two out of three overnight or 24 h urine collections. Urinary albumin was measured centrally by photometric immunohistochemistry at Helsinki University Hospital laboratory (HUSLAB). Normal albumin excretion was defined as AER < 20µg/min or <30 mg/24 h, microalbuminuria as ≥20 and <200µg/min or ≥30 and <300 mg/24 h, macroalbuminuria as ≥ 200µg/min or >300mg/24 h, and end-stage renal disease as dialysis or transplantation (Table 1).

An estimate of the glomerular filtration rate (eGFR) was used to assess kidney function, using serum creatinine that was centrally measured at HUSLAB. eGFR (ml/min/1.73m^2) was calculated by the Chronic Kidney Disease Epidemiology Collaboration equation (CKD-EPI), as presented in Table 9 [136].

Table 9. The CKD-EPI equation for estimating GFR [136].

	Serum Creatinine (µmol/l)	eGFR (ml/min/1.73m^2)
Women (white)	≤62	$141*(sCrea/62)^{-0.329}*(0.993)^{Age}$
	>62	$141*(sCrea/62)^{-1.209}*(0.993)^{Age}$
Men (white)	≤80	$141*(sCrea/80)^{-0.411}*(0.993)^{Age}$
	>80	$141*(sCrea/80)^{-1.209}*(0.993)^{Age}$

8.8. Insulin sensitivity

In patients with T1D, insulin sensitivity was assessed by the estimation of glucose disposal rate (eGDR): eGDR = 24.4 − 12.97 x WHR − 3.39 x AHT − 0.60 x A1C, where WHR stands for waist-to-hip ratio and AHT for antihypertensive treatment and/or blood pressure ≥140/90 mmHg (yes = 1, no = 0) [52,137]. For Study III, insulin sensitivity was calculated as the 24-hour requirement of insulin adjusted for body weight (units/kg body weight).

In non-diabetic subjects, serum insulin concentrations were determined with a Wallac AutoDELFIA Insulin kit (PerkinElmer, Turku, Finland) using an automated analyzer (Wallac 1235 Automatic Immunoassay System, Wallac, Turku, Finland). Blood glucose was determined at HUSLAB. These values were used to calculate insulin sensitivity in non-diabetic subjects using the HOMA-IR index: plasma insulin (µU/ml) x plasma glucose (mmol/l) / 22.5 [138].

8.9. LPS activity

LPS activity reported as endotoxin units (EU/ml) was measured from 1:5 diluted serum samples by the limulus amebocyte lysate assay (LAL, Hycult Biotechnology, the Netherlands). For Studies I and II, an end-point assay was used. In studies III and IV where the samples were lipemic due to postprandial increase in the triglyceride concentrations, the measurement was performed kinetically at 405 nm, so that the sample color development was followed every two minutes for a total of 40 minutes and the minimum absorbance was subtracted from the maximum. In all studies, plate controls were included in the runs and

used for normalization of data between plates. Hence no inter CV is reported, and the intra CV for 0.04 EU/ml was 4.5%.

8.10. The metabolic syndrome

The metabolic syndrome was defined according to the joint statement by Alberti et al. [45]. Participants who fulfilled three or more of the criteria presented in Table 10 were considered to have the metabolic syndrome. Patients with T1D by definition fulfilled the criteria of elevated fasting blood glucose.

Table 10. Criteria for the metabolic syndrome [45]. Fulfilment of three or more of the criteria is regarded as the metabolic syndrome.

Features of the metabolic syndrome	limits
Waist circumference men/women (cm)	≥94/80
Triglycerides (mmol/l) or lipid medication	≥1.7
HDL cholesterol men/women (mmol/l) or lipid medication	<1.0/1.3
Blood pressure systolic/diastolic (mmHg) or antihypertensive medication	≥130/85
Fasting glucose (mmol/l)	≥6.11

8.11. Vascular function – Augmentation Index

When the heart contracts, a forward pulse wave in the arterial wall is created by the left ventricle. However, at sites where the vasculature branches, reflection waves are generated that arrive back at the aortic root during diastole. When the arteries stiffen, the reflection wave arrives back at the central arteries earlier, adding a burden to the forward wave and augmenting the systolic pressure. This phenomenon can be quantified by calculating the augmentation index (AIx, Figure 10) [139]. In Study III, arterial stiffness was measured indirectly by AIx by applanation tonometry from the radial artery of the dominant arm (SphygmoCor, Atcor Medical, Sydney, Australia). By analyzing the difference in amplitude of the second wave P2 (caused by wave reflection) and the first wave P1 (caused by ventricular ejection), the AIx was calculated, which reflects the stiffness of the aorta (Figure 10) [139]. The AIx was corrected for heart rate.

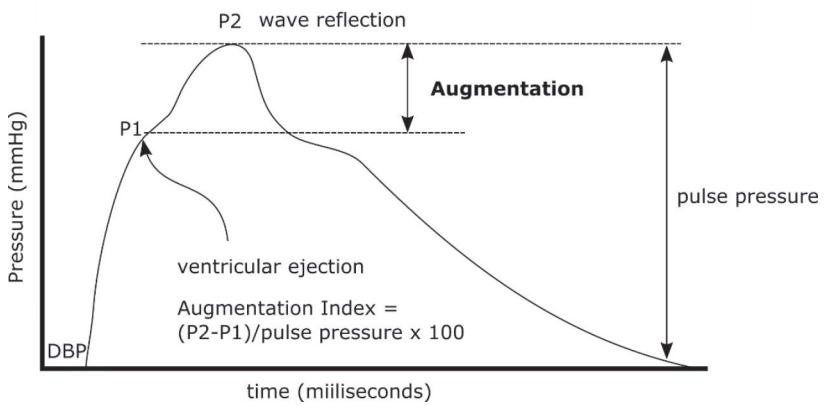

Figure 10. The calculation of the augmentation index (AIx) by pulse wave analysis. The AIx is corrected for heart rate and calculated as a percentage of pulse pressure. DBP, diastolic blood pressure.

8.12. Fecal analytes

Study IV focused on factors associated with gut homeostasis and mainly included the analysis of fecal samples collected in conjunction with the high-fat diet study. The fecal samples were given one week apart, and their mean values were used in the subsequent analysis.

8.12.1. Intestinal alkaline phosphatase

Fecal intestinal alkaline phosphatase (IAP) activity was measured with an in-house method. Fifty milligrams of fecal sample were suspended in 500 µl extraction buffer (0.1 mM ZnCl2, 1 mM MgCl2, 10 mM Tris-HCl pH 8.0) using 0.1 mm glass beads (Precellys, Bertin Technologies, France). After centrifugation (1600 g/10 min/+4°C), supernatants were collected for the determination of fecal IAP activities and total protein concentrations.

A standard curve was prepared using serial dilutions of p-nitrophenyl phosphate (pNPP) and a fixed amount of calf intestinal alkaline phosphatase (CIAP) in the final reactions (Sigma). Substrate stock was prepared by dissolving 1 tablet of pNPP in 5 ml sterile water (4.56 mM). For the standard preparation, an intermediate 2 mM pNPP stock was prepared in an assay buffer (0.1 mM ZnCl2, 1 mM MgCl2, 10 mM Tris-HCl pH 10.0). Standard reactions were performed in a 100 µl volume containing 10 µl pNPP standard (0-20 nmol; 0-67 U/l), 80 µl assay buffer, and 10 µl CIAP (50 U/ml). Sample reactions were performed in a 100 µl volume containing 10 µl fecal sample extracts, 45 µl assay buffer, and 45 µl 4.56 mM pNPP stock. Standards and samples were incubated at +37°C for 30 min, after which the reactions were stopped by adding 20 µl 3M NaOH.

In order to determine the sample background, the fecal extracts and all assay reagents were combined simultaneously into a control well in a total volume of 120 µl before starting the assay. OD values were determined at 405 nm with the correction wavelength set to 630 nm. Samples with high IAP activity were diluted 1:10, 1:50, or 1:200 in the subsequent analyses. The activity of the samples was calculated using the following formula: IAP activity (U/ml) = A/V/T (A is the amount of pNP generated in µmol)/(V is the volume of sample in ml)/(T is the reaction time in minutes). IAP activity was finally normalized with the fecal protein concentrations determined by the Lowry method (DC protein assay, BioRad, California, US). In the present study, the inter-assay and intra-assay CVs for the fecal IAP activity measurements were 13.7% and 2.5%, respectively.

8.12.2. Calprotectin

Concentrations of fecal calprotectin were determined by ELISA according to the manufacturer's instructions (Buhlmann, Swizerland). According to the manufacturer, fecal calprotectin concentrations <50 µg/g in this assay are considered normal. Higher concentrations (50-200 µg/g) implicate increased intestinal neutrophil activity, whereas values >200 µg/g are indicative of active organic disease of the gastrointestinal tract.

8.12.3. Short-chain fatty acids

Short-chain fatty acids (SCFA) were measured in collaboration with Professor Michael Blaut, University of Potsdam, German Institute of Human Nutrition. The SCFAs acetate [C2], propionate [C3], butyrate [C4], valerate [C5], and isovalerate [iC5]) were measured with an HP 5890 series gas chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with an HP-FFAP column (30 m x 0.53 mm; film thickness 1.0 µm) and a flame ionization detector. A total of 300 mg of fresh feces was diluted 1:5 with water and centrifuged at 15000 x g for 5 min. A volume of 23.6 µl 12 mM isobutyric acid (as an internal standard), 280 µl 0.36 M HClO4, and 270 µl 1 M NaOH was added to 50 µl of the

supernatant. The mixture was lyophilized, and the residue redissolved in a mixture of 400 µl acetone and 100 µl 5 M formic acid. After centrifugation, 1 µl of the supernatant was injected into the gas chromatograph. Authentic standards were incorporated in all runs.

8.12.4. Fecal antibodies

Total secretory IgA, IgG, and IgM antibodies and isotype-specific antibodies against oxidized LDL products (copper-oxidized LDL and malondialdehyde acetaldehyde LDL) were measured in fecal samples at Professor Sohvi Hörkkö's Research Laboratory, University of Oulu, Department of Medical Microbiology and Immunology. Human feces were dissolved into ice cold PBS buffer (0.1g feces/1ml buffer). The supernatants were supplemented with protease inhibitors. The concentrations of IgA, IgG, and IgM antibodies binding to oxidized LDL were determined in the supernatants with a chemiluminescence immunoassay [140].

The models of oxidized LDL, copper oxidized LDL (CuOx-LDL), and malondialdehyde acetaldehyde–modified LDL (MAA-LDL) were used in the studies and prepared as previously described [141]. Antigens at 5 µg/ml in PBS-EDTA were incubated overnight at 4°C. Fecal supernatants were incubated for one hour at room temperature (RT), and the amount of antibody bound was detected with appropriate alkaline phosphatase–labeled goat anti-human secondary antibodies for IgA, IgG, and IgM (Sigma cat nos A9669, A3187, A9794) using LumiPhos 530 (Lumigen, cat no P-501) chemiluminescence substrate. The amount of total fecal immunoglobulin in the supernatant was measured using a capture-sandwich chemiluminescent immunoassay, as previously described [140].

8.13. Serum alkaline phosphatase

Serum alkaline phosphatase activity was analyzed at HUSLAB and included the quantitation of total, bone, liver, intestinal, and macromolecular alkaline phosphatase activity. Total alkaline phosphatase in serum was measured photometrically according to the recommendations from the International Federation of Clinical Chemistry (IFCC), using reagent kits and Modular P800 analyzers (Roche Diagnostic).

To identify and quantify AP isoenzymes, the Hydragel 15 ISO-PAL® kit and a semi-automated Hydrasys electrophoresis system (Sebia®, Lisses, France) were used according to the manufacturer's instructions. Shortly, serum samples were run in alkaline-buffered agarose gels (pH 9.4), after which the isoenzymes were stained using a chromogenic substrate 5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium in aminomethyl propanol buffer (pH 10.1). The bands of the isoenzymes were quantified densitometrically. Liver and bone isoforms were separated from each other by lectin treatment.

8.14. ABO blood group and FUT-2 genotype

At the Finnish Red Cross Blood Center, the genetic markers rs8176719, rs8176746, and rs8176747 were examined by TaqMan real-time PCR to determine the *ABO* alleles, as previously described [142].

Fucosyltransferase 2 (FUT2) genotyping was based on the genetic marker rs601338. The primer and probe sequences were defined as described [143]. The distribution of genotype frequencies did not deviate significantly from Hardy–Weinberg equilibrium calculated as $p^2 + 2pq + q^2 = 1$, where p is the frequency of one allele and q the frequency of the other, meaning that their frequency in the population has not been under evolutionary pressure.

8.15. Statistical analysis

The normality of variable distribution was assessed prior to the data analysis with the Kolmogorov–Smirnov test. Normally distributed variables were reported as mean ± standard deviation, non-normally distributed variables as median [25^{th}–75^{th} quartile]. For all studies, a p-value less than 0.05 was considered significant.

In some cases, non-normally distributed variables were ln or log transformed for correlation analyses. Correlation coefficients were calculated by Spearman or Pearson's correlation test as appropriate. Differences in variation between two groups were calculated by Mann-Whitney U test for nonparametric data and by Student's t-test for parametric data. Differences in variation between several groups were assessed by Kruskal–Wallis test for nonparametric data and one-way ANOVA for parametric variables. Differences in frequencies were assessed by Pearson's Chi-squared analysis.

In Study I, variables that were independently associated in a univariate analysis with a progression from normal to microalbuminuria were entered into a Cox regression model (HbA_{1C}, LDL cholesterol, triglycerides, apoB, eGDR, and LPS) to analyze the association with progression.

In Study II, the interaction between LPS activity and features of the metabolic syndrome was tested in a linear multivariable model. Since LPS activity and BMI significantly interacted, the data were split into two groups based on the cut off value for obesity of 25 kg/m^2 (NDC_lean and NDC_ow). LPS or residuals from the LPS/HDL correlation were associated with features of the metabolic syndrome.

In patients with normal AER in Study II, the clinical variables that in the univariate analysis were associated with LPS activity (triglycerides, age at onset of diabetes, diastolic blood pressure, urinary MCP1/urinary creatinine ratio, HbA_{1C}, HDL cholesterol, and waist circumference) were assessed in a multivariable linear regression model.

In Study III, the Wilcoxon signed-rank test was used to compare distributions of one variable at two different time points. Differences in one variable between several time points were assessed with Friedman's test for several related samples. Using partial correlation, the effect of confounding factors was taken into account. In studies III and IV, the area under the curve (AUC) was calculated for variables from the time points between 8:00 and 18:00 with the following formula [$2\ h*((x_1/2)+x_2+x_3+x_4+x_5+(x_6/2))$, where x = value at time point]. For the incremental area (IncA), the area below the first time point * 10 hours was subtracted from the AUC, unless some other time interval is indicated.

All statistical analyses were carried out using the program SPSS (Chicago, Illinois, USA).

9. Results

9.1. Serum LPS activity and the progression of diabetic kidney disease (Study I)

The association of serum LPS activity with kidney disease progression was assessed in 477 patients with T1D, who were followed for 6 years. During this time, 80 out of 239 patients with normal AER developed microalbuminuria, and 79 out of 238 progressed from macroalbuminuria to end-stage renal disease (ESRD). The baseline clinical characteristics of the study participants are presented in Table 4.

Serum LPS activity was markedly increased in patients with macroalbuminuria compared to those with normal AER at baseline (median [IQR]: 53 [38-74] vs. 42 [31-60] EU/ml, $p<0.001$). Patients who developed microalbuminuria during the follow-up time (5.9±2.1 years) had elevated baseline serum LPS activity compared to subjects who remained normoalbuminuric (49 [34-87] vs. 39 [29-54] EU/ml, $p<0.001$). Those that progressed to ESRD showed no difference in baseline serum LPS activity compared to patients who remained macroalbuminuric.

The LPS/HDL ratio reflects the LPS activity with respect to the anti-inflammatory HDL particle, which can bind and neutralize LPS. Both normoalbuminuric (39 [26-65] vs. 30 [21-43], $p<0.001$) and macroalbuminuric progressors (57 [38-82] vs. 47 [28-67], $p=0.017$) displayed an increased ratio at baseline compared to non-progressors (Figure 11).

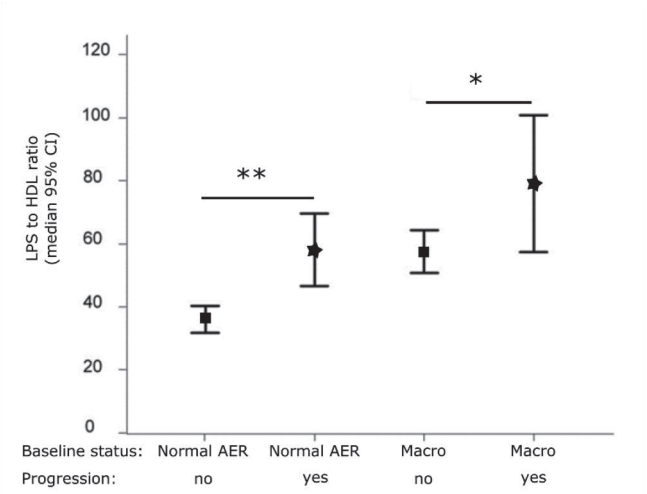

Figure 11. The baseline LPS to HDL ratio in patients with type 1 diabetes according to the progression of diabetic kidney disease. The baseline LPS/HDL ratio was associated with the progression to a more severe kidney status. Macro, macroalbuminuria. Median and 95% CI are shown. ** p<0.001; * p< 0.05.

Variables that in the univariate analysis associated with the development of microalbuminuria (HbA_{1C}, LDL cholesterol, triglycerides, apoB, eGDR and LPS) were tested in a multivariable Cox regression model. HbA_{1C} (hazard ratio 1.28 [95% CI 1.11-1.49], $p=0.001$) and eGDR (0.89 [0.80-1.00], $p=0.044$) were independent risk factors for the development of microalbuminuria. When HbA_{1C} was removed from the model, eGDR (0.83

[0.75-0.91], p<0.001) and lnLPS (1.85 [1.08-3.18], p=0.026) became significantly associated with the development of microalbuminuria.

9.1.1. Study I – Take home message

> In patients with type 1 diabetes, increased serum LPS activity is detected as early as six years prior to the development of microalbuminuria. This indicates that bacterial LPS may contribute to the development of diabetic kidney disease.

9.2. Endotoxin activity and features of the metabolic syndrome (Study II)

The association between LPS activity and features of the metabolic syndrome was assessed in patients with T1D (n = 904) and various degrees of kidney disease as well as in patients with IgA nephropathy (IgAGN; n = 98) and non-diabetic control subjects (NDC; n = 345). Clinical characteristics of the T1D study participants are presented in Table 5, and non-diabetic controls in Table 6.

9.2.1. Patients with type 1 diabetes

The metabolic syndrome was more frequent in patients with T1D and macroalbuminuria (70%) or microalbuminuria (47%) compared to those with normal AER (43%). Patients with macroalbuminuria had a higher LPS to HDL ratio compared to those with microalbuminuria or normal kidney status (macro: 50 [34-77], micro: 37 [29-50], normo: 35 [27-52] EU/ml, p<0.001). LPS activity alone was similarly elevated in patients with macroalbuminuria.

In patients with normal AER serum, LPS activity increased with an increasing number of components of the metabolic syndrome (Table 11).

Table 11. Association between LPS activity and number of features of the metabolic syndrome. Values are compared to patients fulfilling one feature. *p<0.01, **p<0.001.

	1 feature	2 features	3 features	4 or 5 features
LPS (EU/ml)	54 (48-62)	55 (49-63)	57 (51-70)*	78 (62-94)**

To further investigate the effect of a high LPS to HDL ratio, patients with normal kidney status were divided into quartiles. Patients (n=174) in the highest LPS/HDL quartile, compared to those in the lowest quartile (n=145), had higher HbA_{1C}, BMI, waist circumference, serum triglycerides, apoB, and diastolic blood pressure (Table 12). Furthermore, subjects in the highest quartile, compared to the lowest, more often had the metabolic syndrome, decreased insulin sensitivity (eGDR), and a high urinary monocyte chemoattractant protein 1 (uMCP1) to urinary creatinine ratio (uCrea) (Table 12). Similar results were obtained when dividing data by quartiles according to LPS activity instead of the LPS/HDL ratio.

The most significant correlation in the total population of patients with T1D was between the LPS/HDL ratio and serum triglycerides (r=0.73, p<0.001).

9.2.2. Non-diabetic participants

In NDC subjects, the prevalence of the metabolic syndrome was significantly lower than in patients with T1D (48%). The prevalence increased from lean (2%) to overweight (15%) NDC subjects and patients with IgAGN (15%).

Interestingly, elevated LPS/HDL levels were also seen when overweight and lean NDC subjects were compared (45 [35-62] vs. 37 [28-51], $p \leq 0.001$), even when serum LPS activity levels did not differ. This suggests that a low HDL cholesterol level in obese individuals may lead to a prolonged circulation time of LPS in the blood, which could exacerbate inflammation and insulin resistance.

Serum triglyceride concentrations were also significantly associated with the serum LPS/HDL ratio in those without diabetes (all NDC $r=0.51$, $p \leq 0.001$; IgAGN $r=0.58$, $p \leq 0.001$).

9.2.2.1. IgA glomerulonephritis (IgAGN)

In subjects with IgAGN, no association between serum LPS activity and kidney function or AER was observed. When subjects with T1D and IgAGN were matched for sex and eGFR, the LPS activity [62 (50-90) vs. 51 (42-61) EU/ml, $p<0.001$] and the LPS/HDL ratio [43 (30-63) vs. 36 (27-48), $p<0.001$] remained significantly higher in patients with diabetes. Even when subjects were divided into tertiles by fasting serum triglycerides, the LPS/HDL ratio was higher in patients with macroalbuminuria compared to those with IgAGN ($p<0.001$).

9.2.3. Study II – Take home message

> Serum LPS activity is strongly associated with features of the metabolic syndrome. High serum LPS activity in combination with metabolic abnormalities such as insulin resistance and dyslipidemia is likely to increase the risk of developing both microvascular and macrovascular complications.

Table 12. LPS/HDL quartiles in patients with type 1 diabetes and normal albumin excretion rate (Study II).

LPS/HDL quartiles	q1 (<29.0)	q2 (29.0-37.2)	q3 (37.2-50.5)	q4 (>50.5)
N (M/F)	145 (34/111)	148 (61/87)	147 (79/68)	147 (83/64)
Age (years)	48 (40-57)	47 (39-54)	44 (36-55)†	39 (31-47)*
Age at onset (years)	19 (11-28)	16 (10-25)	17 (10-26)	13 (9-21)*
Duration of diabetes (years)	29±13	29±12	27±13	25±10†
HbA_{1C} (%)	7.3±1.6	7.6±1.2	7.7±1.2†	7.9±0.9*
BMI (kg/m^2)	24.4±3.0	25.5±3.5†	25.7±4.0†	26.9±5.5*
Waist (M) (cm)	89±9	92±10	93±10	96±11†
Waist (F) (cm)	80±9	83±10†	83±11†	90±13*
Systolic blood pressure (mmHg)	137±20	136±17	137±17	135±15
Diastolic blood pressure (mmHg)	76±10	77±8	78±9	81±9*
Triglycerides (mmol/l)	0.7 (0.6-0.8)	0.8 (0.7-0.9)†	1.0 (0.8-1.2)*	1.4 (1.2-1.8)*
Cholesterol (mmol/l)	4.7±0.7	4.6±0.7	4.6±0.8	4.8±0.9
HDL cholesterol (M) (mmol/l)	2.1±0.5	1.6±0.2*	1.4±0.2*	1.1±0.2*
HDL cholesterol (F) (mmol/l)	2.0±0.4	1.7±0.2*	1.5±0.3*	1.4±0.3*
ApoA1 (mg/dl)	161±33	145±31*	142±29*	136±29*
ApoB (mg/dl)	64±14	70±13*	77±16*	88±22*
CRP (mg/l)	0.7 (0.3-1.6)	0.7 (0.3-1.4)	1.0 (0.4-2.1)	1.2 (0.4-3.5) †
eGFR ($ml/min/1.73m^2$)	97 (84-108)	100 (92-109)†	103 (93-115)†	106 (93-115)*
eGDR (mg/kg/min)	7.4±2.3	7.0±2.3	6.4±2.3*	6.1±2.3*
uMCP1/uCrea ($pg*ml^{-1}/\mu mol*l^{-1}$)	13 (8-23)	14 (9-23)	15 (9-25)	16 (10-30)†
Metabolic syndrome (%)	22	38†	42*	69*
AHT medication (%)	35	26	33	32
Lipid medication (%)	23	16	21	26
Smoking (%)	10	13	18†	25†

All data are compared to quartile 1. eGFR, estimated glomerular filtration rate; AHT, anti-hypertensive medication; uMCP1/uCrea, urinary monocyte chemoattractant protein 1/urinary creatinine. †$p<0.05$, *$p<0.01$.

9.3. Study III: High-fat diet and vascular dysfunction

The acute effect of three high-fat meals on endotoxemia, inflammation, lipid metabolism, and vascular function was studied in 34 non-diabetic controls and 37 uncomplicated patients with T1D. At fasting, patients with T1D and controls were of similar age, sex, BMI, lipid profile, and blood pressure; see the clinical characteristics (Table 7).

9.3.1. Endotoxemia

Levels of fasting serum LPS activity and LPS area under the curve (LPS-AUC) following the three high-fat meals did not differ between patients with T1D and controls [fasting 0.82 (0.57-1.43) vs. 0.90 (0.52-1.24) EU/ml and AUC 9.8 (6.8-14.1) vs. 8.8 (6.2-11.3) EU/ml]. Three consecutive meals had only a modest effect on postprandial LPS activity, as can be seen in Figure 12.

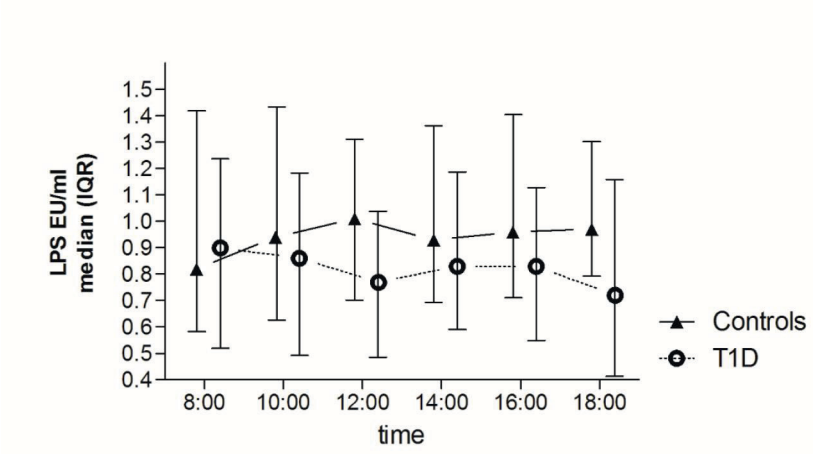

Figure 12. The effect of three high-fat meals on serum LPS activity levels. Non-diabetic controls and patients with type 1 diabetes (T1D) are indicated with filled triangles and open circles respectively. Lines indicate median and whiskers 25th to 75th quartile. Meals were given at 8:00, 12:00, and 16:00.

In patients with T1D, fasting LPS and LPS-AUC correlated highly with CRP concentrations (fasting r = 0.428, p = 0.009; AUC r = 0.338, p = 0.044) and daily insulin dose (fasting r = 0.479, p = 0.004; AUC r = 0.528, p = 0.001).

9.3.2. Inflammatory markers

Serum IL-6 concentrations increased during the study day and peaked at 16:00. Compared to the fasting level, the increase was 610% in patients with T1D and 570% in controls. However, no association between serum IL-6 concentrations and LPS activity was observed. Other inflammatory markers (CRP, SAA, sCD14) varied only modestly during the day, and the levels were similar between controls and patients with T1D. Leukocyte and neutrophil counts increased significantly during the study day comparing fasting and post-prandial samples at 8:00 and 18:00; however, in patients with T1D this increase was blunted: leukocyte change (T1D vs. control 114 vs. 128%, p=0.022) and neutrophil change (118 vs. 147%, p=0.038).

9.3.3. Lipid metabolism

Fasting triglyceride concentrations were similar in patients and controls. However, apoB-48, a specific chylomicron marker, was higher both at fasting [5.0 (3.7-8.8) vs. 3.8 (2.7-5.4) mg/dL, p = 0.014] and postprandially in T1D patients [AUC 109 (7-166) vs. 89 (51-128), p = 0.035]. Since chylomicrons have been shown to be involved in the transport of LPS from the intestine to the general circulation [91,144], we tested the association between apoB-48-AUC and LPS-AUC; however, no correlation was observed. Only after breakfast was the incremental area (IncA 8:00-12:00) of apoB-48 associated with LPS-IncA (r = 0.351, p = 0.036). ApoE is a chylomicron turnover regulator, and the levels were lower in T1D patients compared to controls at fasting [20.2 (12.1-25.3) vs. 24.4 (18.8-32.4 mg/dl), p = 0.022] and postprandially [AUC 194 (144–253) vs. 260 (202–330), p = 0.001].

NMR analysis was used to determine the distributions of HDL particles at fasting. Despite similar HDL cholesterol concentrations, patients with T1D had more large HDL particles (14.3 nm) [$441*10^{-7} \pm 176*10^{-7}$ vs. $309*10^{-7} \pm 216*10^{-7}$ nmol/l; p = 0.007] than controls. Serum lipid transfer proteins involved in HDL modeling were elevated in patients with T1D compared to controls: PLTP-AUC [67910 (58431–75158) vs. 51315 (47079–59470), p < 0.001] and CETP-AUC [280 (233–313) vs. 250 (204–278), p = 0.007]. On the other hand, paraoxonase (PON-1)-AUC, an anti-oxidative enzyme bound to HDL, was lower in patients with T1D [191 (135–470) vs. 463 (167–678), p = 0.027].

9.3.4. Arterial stiffness

The augmentation index (AIx) did not differ between patients with T1D and NDC at fasting. Although a postprandial relaxation of the arteries was observed in the controls (p for trend 0.029), this phenomenon was not evident in the patients (Figure 13). In patients with T1D, glucose variability associated with AIx at all time points 8:00 (r = 0.420, p = 0.017), 12:00 (r = 0.392, 0.048), and 16:00 (r = 0.434, p = 0.013). No association between LPS and AIx was observed.

9.3.5. Study III – Take home message

A postprandial increase in endotoxin levels was not evident after three consecutive high-fat meals. However, patients with type 1 diabetes showed an adverse atherogenic lipid profile (high levels of triglyceride-rich remnants and the low antioxidative capacity of the HDL molecule) as well as an impaired postprandial vascular relaxation response. These changes may render the patients at risk of cardiovascular disease.

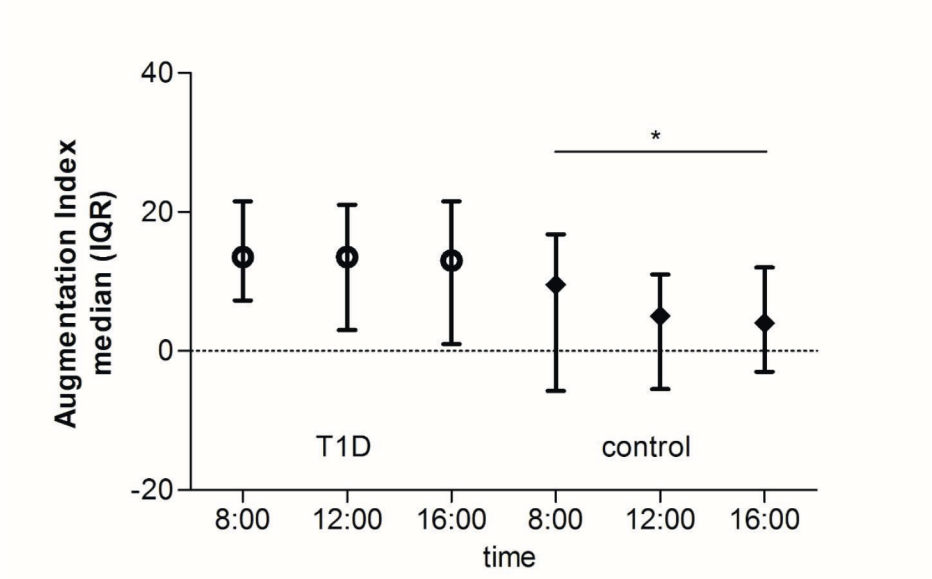

Figure 13. The postprandial augmentation index in patients with type 1 diabetes (T1D, circles) and non-diabetic controls (diamonds). Symbols indicate median and whiskers 25th to 75th quartile. * p for trend <0.05.

9.4. Study IV: Intestinal inflammation in type 1 diabetes

Factors involved in gut homeostasis (fecal intestinal alkaline phosphatase, short-chain fatty acids, secretory antibodies, and calprotectin concentrations) were assessed in 41 non-diabetic controls and 46 patients with T1D (36 with normal AER and 10 with macroalbuminuria).

9.4.1. Fecal protective factors

Fecal intestinal alkaline phosphatase (fIAP) concentrations were lower in T1D patients with normal AER compared to non-diabetic controls (61 [26-221] vs. 131 [73-837] U/l, p=0.01) (Study IV, Figure 1). Fecal IAP activities were not associated with serum LPS activity, nor with lipids or CRP concentrations.

Total fecal short-chain fatty acid concentrations (SCFAs) were lower in patients with T1D and normal AER compared to controls (414 [234-638] vs. 496 [404-720] mmol/g dry weight, p=0.043), and so were propionate (70 [33-106] vs. 91 [66-116] µmol/g dry weight, p=0.015) and butyrate concentrations (39 [23-81] vs. 71 [40-101] µmol/g dry weight, p=0.020) (Table 13).

The total amounts of fecal IgA, IgG, and IgM as well as oxLDL specific antibody concentrations were assessed by indirect ELISA. Total IgG and IgM concentrations were similar between the groups. However, total IgA concentrations were lower in patients with normal AER compared to controls (1.9 [0.7-3.6] vs. 3.4 [1.5-6.9] µg/g wet weight, p=0.015). A significant positive correlation in the total population was observed between fecal IAP and total immunoglobulin concentrations: IgA vs. fIAP (r=0.266, p=0.039), IgG vs. fIAP (r=0.349, p=0.001), and IgM vs. fIAP (r=0.432, p<0.001).

Table 13. Fecal short-chain fatty acids. The total SCFAs, proprionate, and butyrate were lower in patients with T1D and normal AER compared to controls. Patients with macroalbuminuria did not differ from those with normal AER. dw, dry weight * p<0.05.

	Controls (n=41)	Normal AER (n=36)	Macroalbuminuria (n=10)
Total SCFAs (mmol/g dw)	496 (404-720)	**414 (234-638)***	318 (159-615)
Acetate (µmol/g dw)	298 (252-457)	258 (160-412)	211 (131-389)
Proprionate (µmol/g dw)	91 (66-116)	**70 (33-106)***	47 (16-108)
Butyrate (µmol/g dw)	71 (40-101)	**39 (24-81)***	30 (10-52)
Isovalerate (µmol/g dw)	19 (15-23)	19 (16-22)	20 (9-23)
Valerate (µmol/g dw)	13 (9-16)	10 (8-14)	9 (4-17)

Patients with normal AER are compared to controls, and those with macroalbuminuria to subjects with normal AER.

OxLDL specific antibodies were also lower in patients with normal AER compared to controls: IgA-MAA (9.2 [3.8-28.8] vs. 24.4 [12.7-57.1] relative units (RU, p=0.008) and IgA-CuOx (22.1 [12.0-43.4] vs. 56.4 [23.8-112.1] RU, p=0.002). Patients with macroalbuminuria displayed lower concentrations of IgA-MAA compared to those with normal AER (0.7 [0.4-10.9] vs. 9.2 [3.8-28.8] RU, p=0.043).

9.4.2. Fecal inflammatory factors

Calprotectin was elevated in the stool of patients with T1D and normal AER compared to non-diabetic controls (48 [29-90] vs. 29 [16-59] µg/g, p=0.028) (Study IV, Figure 1). Patients with macroalbuminuria had similar fecal calprotectin concentrations as those with normal AER. A mild pro-inflammatory state of the intestine, with fecal calprotectin >50µg/g, was more frequent among patients with normal AER than in controls (50% vs. 25%, p=0.024). Moreover, high concentrations of fecal calprotectin >200µg/g, a criteria for organic inflammatory bowel disease, were more frequent in macroalbuminuric patients compared to those with normal AER (40% vs. 6%, p=0.017).

9.4.3. Study IV – Take home message

> Patients with type 1 diabetes displayed a lack of intestinal protective factors (fecal IAP, short-chain fatty acids, and antibodies against atherosclerotic oxLDL compounds) and an increase in gut inflammation. A disruption in gut homeostasis may contribute to the development of inflammation-driven conditions such as insulin resistance, nephropathy, and cardiovascular disease. Patients with type 1 diabetes and gut inflammation could potentially benefit from the clinical use of IAP or the upstream regulator butyrate in their treatment.

10. Discussion

The main findings of this thesis show that high serum LPS activity preceded the development of microalbuminuria, and that serum endotoxin concentrations were associated with features of the metabolic syndrome. No increase in serum LPS activity was observed in response to high-fat meals. Postprandially, the patients with T1D displayed unfavorable lipid profiles and stiffer arteries – putative risk factors for CVD. Lastly, we show that the gut in patients with T1D is more inflammation prone and has a disturbed homeostasis, including lower intestinal alkaline phosphatase concentrations.

10.1. Patient selection

The participants with T1D in all studies from I to IV took part in the FinnDiane Study, a prospective study of patients with T1D. The total number of patients in FinnDiane is currently about 5000, which comprises about 13% of the patients with T1D in Finland (Finnish Diabetes Association homepage, 13.8.2015).

The FinnDiane Study centers consist of all university hospitals, central hospitals, and district hospitals as well as large primary health care units throughout Finland. The hospitals currently mainly take care of patients with more complications. The primary health care centers involved in FinnDiane constitute about 11% of all heath care centers in Finland and are sites for the care of young patients with T1D without complications. As FinnDiane has a special focus on the enrollment of patients with kidney problems, the above facts could result in an overrepresentation of patients with renal complications in the study compared to the general type 1 diabetic population. However, given the large number of patients enrolled throughout the nation, the FinnDiane Study is a representative cohort of patients with T1D in Finland.

Studies III and IV are subject to potential selection bias, since individuals interested in their personal health were more likely to participate in the high-fat diet study. This is probably reflected in the better glycemic control as well as better socioeconomic status of the participants. This would, however, probably only diminish the differences between the patient groups and non-diabetic controls, rather than make the results less reliable.

Studies II, III, and IV are cross-sectional in their design, which means that one must be cautious when assessing the associations, since other confounders may affect both parameters. Further, in a cross-sectional setting, “the chicken and the egg” causality dilemma remains unsolved. Study I followed the study subjects for about six years to determine their renal outcome. This setting allows the estimation of the predictive value of risk factors related to the progression of renal events.

10.1.1. Sample storage

Long-term storage of serum at -20ºC, especially in Studies I and II, may have adverse effects on the stability of peptides and proteins. However, the vast majority of the analytes, such as creatinine, lipids, lipoproteins, CRP, and HbA_{1C}, used in the studies were measured within a short timeframe in relation to the sampling. Prolonged storage time may increase protein degradation, which could influence the results. Nevertheless, this would be likely to weaken the associations in the data, rather than strengthen them.

To test the effect of a prolonged storage time on LPS activity, we measured 2056 serum samples with a storage time ranging from 4 months to 19 years and plotted it against the

sample storage time (Figure 14). Samples that had been stored longer than 13 years had arbitrarily high LPS activity. The serum samples in studies I–IV were stored <10 years.

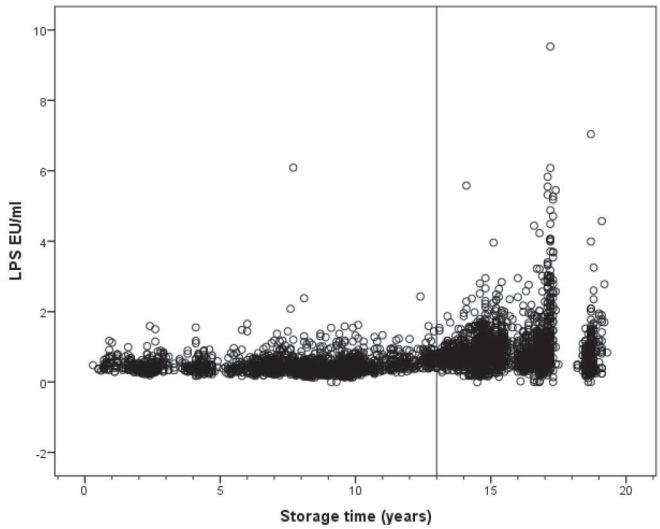

Figure 14. LPS activity (EU/ml) in serum samples plotted against storage time (years). The vertical line is set at 13 years, up to which point the samples were unaffected by storage time.

10.1.2. Renal status

The classification of renal status in the FinnDiane study relies on three consecutive timed urine collections. In Study III, the patients with microalbuminuria (8 out of 37 patients) were pooled together with those with normal AER and compared to the non-diabetic controls. According to data from the DCCT, 47% of the patients with T1D and microalbuminuria progressed to macroalbuminuria, a lower GFR, or ESRD during a follow-up of 10 years, and around 40% regressed to the range of normal AER [145]. This could indicate that even though the patient population in Study III is more heterogeneous, the patients with microalbuminuria are not likely to affect the results considerably. Further, the greater number of subjects gives more power to the statistical analysis.

10.1.3. Insulin sensitivity

Insulin sensitivity can be directly measured by the euglycemic hyperinsulinemic clamp technique. Based on clamp studies in patients with T1D, an estimated glucose disposal rate (eGDR) was generated by a mathematical equation using waist-to-hip ratio, HbA_1, and the presence or absence of hypertension [137]. After this publication, the eGDR has been widely used to indirectly assess insulin sensitivity in patients with T1D, and in our studies, the formula was modified for the use of HbA_{1C} instead of HbA_1 [52]. However, in the original publication of Williams et al., the eGDR equation explained 57% of the true glucose disposal, indicating that other factors account for 43% of the GDR [137]. Another method for the assessment of insulin sensitivity in type 1 diabetic patients has been daily insulin dosage adjusted for body weight. This also correlated well with insulin sensitivity in the study of Williams et al. [137].

In studies I and II, insulin sensitivity was estimated by eGDR. In Study III, the daily insulin dose adjusted for body weight was used as an estimate of insulin sensitivity in correlations with serum LPS activity (Study III). Increases in body weight have adverse effects on insulin sensitivity in patients with T1D and T2D [146,147], and higher insulin requirements could

be considered a surrogate marker of insulin resistance. Insulin dose did not correlate with HbA_{1C} concentrations (Study III), indicating that across daily insulin doses, similar long-term glucose concentrations were achieved. This suggests that the difference in the insulin dose reflects a difference in insulin sensitivity. Why eGDR performs better at some times and the insulin dose at other times may have to do with the fact that eGDR was developed using data from patients with uncomplicated T1D. Yet since the majority of the participants in Study III were complication free, the reason for the superior performance of the insulin dose in the analyses still remains an open question.

10.2. Can endotoxins affect the development of kidney disease?

The mechanisms by which endotoxins are able to affect the development of kidney disease likely arise from the ability of LPS to cause podocyte foot process effacement, downstream of the TLR4 receptor. In addition to podocytes, TLR4 is also expressed by the tubules and tubulointerstitial cells [71,72]. As discussed in the introduction (6.5 Mechanisms of endotoxemia-induced kidney injury), some pathways, including catepsin L, uPAR, and B7-1, have been described as causative for LPS-induced proteinuria [9-11]. However, by up-regulating inflammatory genes, LPS is likely to also affect the insulin signaling of the podocyte, possibly leading to insulin resistance and apoptosis [148]. In fact, insulin resistance has been strongly linked to the progression of kidney dysfunction in patients with T1D [52]. In line with this, the anti-apoptotic factor PDK-1 was down-regulated in podocytes treated with human sera with high LPS activity [125].

In Study I, we show that high serum LPS activity precedes the development of microalbuminuria. This indeed indicates that low concentrations of LPS may exacerbate kidney injury already at early stages of disease development. The fact that the LPS/HDL ratio, but not LPS alone, was associated with the progression of macroalbuminuria to ESRD also signifies the role of dyslipidemia at later stages [149].

Is LPS activity then associated with all forms of proteinuric kidney disease? We sought to answer this by comparing the patients with T1D and macroalbuminuria to the patients diagnosed with IgAGN in Study II. The patients with T1D and macroalbuminuria, compared to those with IgAGN, displayed an elevated LPS/HDL ratio. This fact could not be explained by impaired kidney function, since kidney function remained the same when the groups were matched for sex and eGFR. Moreover, in the IgAGN population, LPS did not correlate with either AER or eGFR, unlike in the patients with T1D. Hence, LPS does not in these cross-sectional analyses seem to be involved in the renal impairment associated with IgAGN.

Cross-sectionally in Studies I and II, patients with macroalbuminuria had higher LPS activity than those with normal AER; however, since LPS had no effect on the progression to ESRD, it may be that other factors become more important at more advanced disease stages and confound the effect of LPS. Moreover, no studies exist on the variability of circulating LPS activity in humans over time, leaving the question open of whether LPS is potentially an initial insult or is chronically elevated in DN. Interestingly, unpublished data from FinnDiane suggest that once the eGFR starts to decline, the slope remains stable until the development of ESRD.

10.3. What is the contribution of LPS to the metabolic syndrome?

In animal studies, metabolic endotoxemia was described as an initiator of obesity and insulin resistance [94]. As obesity and insulin resistance are common features not only in type 2 diabetes but also in patients with type 1 diabetes, a shared etiology between the two forms is possible [46,52].

In a series of studies, Cani et al. showed that lean mice on a high-fat diet (72% of total calories) for four weeks [94,150] had modestly elevated LPS activity. The same was evident in genetically obese mice on normal chow. The elevation in endotoxins was associated with increased fat deposition, systemic and tissue-specific inflammation, and insulin resistance, and the phenotype was reproduced with a low dose infusion of LPS in lean mice on normal chow [94,150]. Moreover, lean mice lacking the CD14 co-receptor for LPS were resistant to weight gain induced by a high-fat diet, tissue-specific inflammation, hepatic lipid deposition, and insulin resistance, indicating TLR4 activation by LPS as a mediating event in high-fat diet–induced inflammation and metabolic dysfunction [94]. Increased TLR4 expression in the adipose tissue and skeletal muscle has been reported in obese subjects (reviewed in [151]).

Importantly, differences in gut microbiota are evident, including obesity associated changes in biodiversity compared to lean individuals. Specifically, a lower abundance of *Bifidobacteria* has been associated with obesity, and in rodents their abundance was associated with gut barrier function and reduced intestinal endotoxin levels [151].

In Study II, we showed that non-diabetic overweight individuals had an elevated LPS/HDL ratio (even with similar LPS activity levels) compared to lean individuals. Moreover, the association between LPS activity and fasting triglycerides, insulin, and CRP concentrations was more pronounced in those that were overweight. In the patients with T1D and normal AER, a positive association between the number of fulfilled features of the metabolic syndrome (metabolic score) and LPS activity was observed. Importantly, a high LPS/HDL ratio was associated with decreased insulin sensitivity in patients with T1D and with high fasting insulin in the non-diabetic controls.

The acute effect of high-fat feeding on circulating endotoxins was evaluated in Study III; however, in this setting no increase in postprandial endotoxemia was observed in the patients with T1D and normal AER or in the non-diabetic controls. This observation may be due to the fact that patients with T1D but without renal complications are resistant to the effects of high fat, whereas those with a genetic propensity to renal complications are vulnerable to the effect of a high-fat diet.

10.4. What is the origin of circulating LPS?

Many potential entrance routes for endotoxins are possible and include the oral cavity/periodontitis, the intestine, insulin injection sites, and other local or systemic infections. Notably, bacterial infections are more frequent in patients with T1D, and the recurrent use of antibiotics is associated with an increased risk of incident microalbuminuria [152]. We sought to answer the question of the origin of LPS in studies I, III, and IV.

10.4.1. Is serum LPS derived from oral bacteria?

Previous studies have shown a close association between periodontitis and diabetes on one hand and periodontitis and kidney disease on the other [7,153-155]. We assessed this association in our patients with T1D by analyzing the serum antibodies to two common periodontal pathogens *P. gingivalis* and *A. actinomycetemcomitans*. However, the antibody concentrations were not associated with kidney disease progression (Study I). Still, this does not rule out the possible contribution of the oral cavity and some other pathogens to circulating LPS and the development of nephropathy. In a recent meta-analysis, the pooled odds ratio for the association of periodontitis and chronic kidney disease was 1.65 in cross-sectional studies. In interventional studies, where the effect of periodontal treatment on eGFR was assessed, all studies showed a positive effect on kidney function [110]. This suggests that oral health and kidney disease share common underlying pathogenic mechanisms.

10.4.2. Is serum LPS derived from intestinal bacteria?

The gut contains a large pool of gram-negative bacteria. In fact, the microbial cells of the intestine exceed 10-fold the number of somatic cells in the human body and thus represent a potentially massive source of endotoxins [12]. Since endotoxins are lipid soluble and their uptake from the intestine has been linked to chylomicron metabolism [92], we explored the role of a high-fat diet on endotoxemia in Study III.

Surprisingly, the high-fat diet had little effect on the circulating endotoxins in our study. The question remains of whether this is due to the study design, which used acute exposure, or to fat composition, or if some other chronic condition such as insulin resistance or gut inflammation is needed for an increase in endotoxemia. In a recent study, the association of LPS to chylomicron triglycerides was evaluated in lean (BMI 20-25 kg/m^2) and obese subjects (BMI 30-35 kg/m^2), and a positive postprandial correlation between the two was evident only in obese subjects [156]. This indeed indicates that chronic metabolic conditions may be required for a change in postprandial endotoxemia. Recently, the association between LPS and nutrient intake/CVD was assessed in a large population-based study. Even though LPS was associated with energy intake, the association of LPS to coronary heart disease was independent of nutrient intake [157]. This indicates that other routes, apart from the nutrient-induced uptake of LPS from the intestine, may be associated with detrimental health consequences [157]. Taken together, the question of where LPS comes from remains unanswered. Nonetheless, the present data cannot rule out either the oral cavity or the gut microbiome as potential sources of endotoxins.

10.5. The LAL assay

The fact that the LPS measurement is based on blood from the horseshoe crab means that batch to batch variations or crab to crab variation can arise. This also prevents the LAL assay from being used clinically in hospitals. To overcome this problem, we used one specific lot for all measurements in each publication; however, a direct comparison of LPS activity levels between publications is not possible. Furthermore, the non-standardized treatment of blood samples (chemical treatment, heat inactivation, dilution) to reduce the interference of blood factors has led to very variable reports on endotoxin concentrations [151]. The yellow color formation of the LAL assay is measured at 405 nm, which also is problematic since both plasma and serum samples have a yellow coloring. In our assays, the samples were diluted 1:5 in endotoxin free water prior to analysis. As stated, the read-out from the LAL

assay is LPS activity, which is the amount of LPS in the circulation that has biological activity, not the total concentration of LPS that is bound and inhibited by other blood components.

New LAL-independent methods of measuring LPS include an antibody-based detection system where IgM antibodies bind the most well-conserved structural component of the LPS molecule, lipid A (Spectral Medical Inc, web page 14.8.2015, www.spectraldx.com). However, this system is blood based, and even when lipid A is relatively well conserved, modifications in its structure are evident between bacterial strains [58]. Cell-based assays to measure LPS-induced cytokine responses are not well suited for large sample numbers and small volumes. Recently, mass spectral analysis of LPS-derived 3-hydroxymyristate was described; however, this method quantifies total plasma LPS concentration but not the biological activity [158]. To conclude, there are no superior methods to the LAL assay at the moment. Nevertheless, care must be taken since sample turbidity and hemolysis give false positive results in the LAL end-point assay. In Studies I and II, fasting samples were used, and the end-point assay was utilized. In Studies III and IV, a kinetic measurement was conducted to overcome the issue of sample turbidity caused by an increase in triglycerides. All hemolytic samples were omitted from the subsequent analyses.

In studies I and II, the LPS activity values were multiplied by 100, which was based on an older procedure not used later in studies III and IV. In Studies I and II, LPS activity alone or the LPS/HDL ratio were used as outcome measures. What then is the correct way to report LPS activity? LPS activity alone has been shown to be a strong predictor of incident type 2 diabetes [7]. However, it can be argued that since the HDL particle is mainly responsible for detoxification [78], it is relevant to report LPS activity with respect to HDL concentrations. On the other hand, HDL cholesterol is also included in the definition of the metabolic syndrome [45]. Thus, in studies I and II, we performed the analysis with both the LPS and LPS/HDL ratios, as well as the residuals from the LPS/HDL correlation (Study II). For Studies III and IV, we chose to report only kinetic LPS activity measures.

10.6. Endotoxins, inflammation, and complications

Although this thesis has mainly focused on bacterial endotoxins, it is important to keep in mind that the TLR4 has also other endogenous (e.g. free fatty acids) and exogenous ligands (e.g. dietary fatty acids). It is likely that during high-fat feeding, inflammation and insulin resistance can be caused both by endotoxins as well as fatty acids through the activation of TLR4 and the inhibition of insulin receptor signaling [159]. Indeed, we observed no postprandial increase in LPS activity in Study III. Yet germ-free mice or mice treated with antibiotics specific for gram-negative bacteria do not develop high-fat diet–induced insulin resistance, indicating a central role of the gut microbiota in the inflammatory process [159].

Some controversy exists on how the entrance route of endotoxins affects the inflammatory response. The drawback in many studies lies in drawing parallels between endotoxemia induced by a high-fat diet and LPS intravenous injection (reviewed by [100]) or the subcutaneous administration of LPS [94]. Endotoxins originating from the gut may have a diminished inflammatory potential compared to intravenous injections due to the cleavage of phosphate residues from the core polysaccharide by intestinal alkaline phosphatase (IAP) [74]. We and others have reported that circulating inflammatory factors (IL-6, CRP, SAA, and sCD14) were not associated with LPS activity postprandially after high-fat meals

[156,160,161]. This may also be due to differences in study design or qualitative differences in the saturation degree of fats.

From Studies I to IV, insight was gained on endotoxins and other factors that may contribute to an elevated risk of cardiovascular complications in patients with T1D. In a recent Finnish population-based follow-up study, LPS predicted incident coronary heart disease independently of obesity, diabetes, CRP, sex, and cholesterol [157]. Cardiovascular mortality is increased in patients with uncomplicated T1D, and DN is associated with an even higher risk of death from CVD [26]. Our studies (I–IV) show a few mechanisms by which the atherogenic load may be increased in patients with T1D. Firstly, LPS is a potent driver of inflammation, which is tightly linked with CVD in T1D [51]. Secondly, atherogenic modifications of lipid-associated parameters in the patients included a reduced antioxidative capacity of the HDL molecule, elevated circulating chylomicron remnants, and finally lower protection against the uptake of oxidized lipids from the gut. Both chylomicron remnants and oxidized LDL affect the vasculature and are involved in the development of vascular plaques [162-164]. The oxLDL concentrations have been shown to be elevated in patients with T1D and correlate with the thickening of the carotid intima media, that is, the innermost two layers of the arterial wall, a process associated with atherosclerosis [165].

The observation that patients with T1D show impaired vasodilation in response to high-fat meals (Study III) further supports that they are at an increased risk of cardiovascular disease. This may be related to intestinal function and to the absorption and processing of lipids. Interestingly, pharmacological studies have demonstrated that the oxidation of chylomicron remnants significantly enhances their inhibitory effects on endothelium-dependent vascular relaxation and potentiate vasoconstriction in rat and pig arteries [163]. Traditional factors affecting vascular function include insulin, which acts as a vasodilator that increases nitric oxide production in the endothelium and induces the relaxation of the surrounding smooth muscle tissue [14]. High glucose, on the other hand, induces the generation of oxygen radicals from the mitochondria and an increased production of inflammatory cytokines [37], which can cause vasoconstriction. Interestingly, oscillating glucose concentrations in both healthy individuals and subjects with type 2 diabetes are associated with worse endothelial function than constant hyperglycemia [166]. Since no association was seen between the augmentation index and either insulin dose or inflammatory factors in patients with T1D, the persistent elevation in chylomicron remnants as well as the association with glucose variability during the study day could potentially explain the high AIx (Study III).

10.6.1. Does intestinal alkaline phosphatase protect from endotoxemia?

Increased permeability of the gut is evident in patients with T1D [113-116]. Whether this is due to changes in gut microbiota remains unknown [167]. Intestinal alkaline phosphatase is essential for gut homeostasis and protects from both intestinal infection and chronic inflammation. Moreover, it can cleave phosphate residues from the bacterial LPS molecules, mitigating their inflammatory capacity, but IAP is also involved in the regulation of fat transport through the intestinal enterocytes [75].

In Study IV, we showed that the protective factors involved in gut homeostasis were lower in patients with T1D and normal AER compared to non-diabetic controls. These included lower IAP and butyrate concentration in feces. Of note, the gut inflammatory marker calprotectin was also elevated in the patients with T1D. A curious question that arose in Study III was why the postprandial neutrophil increase was blunted in the patients compared

to the non-diabetic controls. Although the question was not answered by Study IV, it can be speculated that the neutrophils may be homed to the inflamed gut of the patients, which can be seen as an increase in neutrophil-derived calprotectin in the feces of the patients with T1D. Furthermore, severe intestinal inflammation was evident in four out of ten patients with macroalbuminuria, posing the possibility that T1D/DN and inflammatory bowel disease share a common etiology, such as reduced IAP activity, or affect each other [75,168]. Other inflamed tissues, such as white adipose tissue, may also play a role in neutrophil homing [169].

Intestinal alkaline phosphatase is decreased during fasting in humans and can be restored upon feeding [170]. The molecule is important in maintaining the gut barrier; an increased bacterial translocation from the gut was demonstrated in intravenously fed mice [171]. It has been suggested that IAP maintains the barrier function by blocking intestinal inflammatory factors and by maintaining tight junctions in the gut epithelia [170]. This idea, together with our findings on the involvement of LPS in the development of diabetic nephropathy (Study I) on one hand and lower fecal IAP concentrations on the other (Study IV), suggests an interesting hypothesis that these two phenomena are linked. Both low fecal IAP [172,173] and elevated circulatory LPS activity [174] have been reported in patients with IBD. As to why no association was observed between serum LPS activity and fecal IAP concentrations in Study IV, it can be speculated that (1) higher serum IAP in patients may result in increased LPS dephosphorylation and (2) albeit the lower fecal IAP concentrations in patients, gut integrity may still be preserved.

10.7. Can diabetic complications be prevented by IAP?

A mechanism to prevent the metabolic syndrome and obesity or to restore insulin sensitivity in animal experiments on high-fat feeding is the supplementation of intestinal alkaline phosphatase, a known regulator of gut homeostasis [175]. Since insulin sensitivity is tightly linked with blood glucose control and inflammation, as well as both microvascular and macrovascular complications, IAP could have potential therapeutic effects [42,48,75].

IAP can directly modulate the intestinal microbiota in a favorable commensal direction, at least in murine models, by reducing luminal adenosine triphosphate (ATP) and other triphosphate concentrations that inhibit commensal bacteria [75,176]. Clinical trials using IAP have shown promising results in the short-term improvement of ulcerative colitis [75]. Furthermore, in septic patients, IAP administration has led to improved renal function [177,178].

Whether IAP could be used to improve insulin sensitivity and thus prevent the metabolic syndrome in patients with diabetes, thereby possibly reducing the incidence of diabetic complications in the long run, remains a fascinating open question.

11. Summary

Taken together, the data presented in this thesis suggest that bacterial endotoxins are associated with the development of DN (Study I), but also with features of the metabolic syndrome (Study II). As the metabolic syndrome itself is a risk factor for DN and cardiovascular disease, the effect of endotoxins may partly arise not only from direct disruption of the filtration barrier, but also from indirect mechanisms involving insulin resistance both at the tissue as well as the systemic levels. No direct evidence of LPS accumulation from the gut due to an acute high-fat diet was observed, even though atherogenic lipid modifications and an altered vascular response were evident in the patients with T1D (Study III). Finally, we showed that the gut is prone to inflammation in patients with uncomplicated T1D, and that protective factors, such as intestinal alkaline phosphatase, the short-chain fatty acid butyrate, and antibodies to oxLDL products, are low (Study IV). This most likely is accompanied by changes in the gut microbiota and could cause a shift in composition leading to higher gut permeability and an increase in endotoxins in the circulation.

12. Future prospects

In the autumn of 2015, serum samples from all FinnDiane participants were measured for LPS activity, which means that the potential is huge for LPS to be used as a predictive biomarker for other complications such as cardiovascular disease. Furthermore, this analysis gives us the possibility to perform a genome-wide association study using LPS as a quantitative trait. This would allow us to study which genetic markers contribute to elevated endotoxin levels. In addition, studies using Mendelian randomization are of interest for pointing out causalities between genetic variants, LPS, and clinical outcome measures. Studies that may illuminate the entrance route of systemic endotoxins are also underway and include the analysis of periodontal pockets and the association of bacteria with diabetic kidney disease. Finally, we are in the process of evaluating the contribution of LPS to metabolically harmful visceral fat.

13. Acknowledgements

This work was carried out at the Division of Nephrology, Department of Medicine, at the University of Helsinki and Helsinki University Central Hospital, at the Folkhälsan Research Center, and at the Research Programs Unit (Diabetes and Obesity) at the University of Helsinki.

Docent Markku Lehto and Professor Per-Henrik Groop were the supervisors of this doctoral thesis. I would like to express my warmest gratitude to Markku for his support, guidance, and help during this work. He has been inspirational with regard to the science itself, but has also shown great warmth and empathy in matters outside of science. I also sincerely thank Perra for always having a clear greater picture and finding the time, regardless of his endless travels, to support and engage in scientific discussions and supervision. Carol Forsblom is also thanked for his friendly and supportive attitude; even if he was not an official supervisor, he has always found the time to answer my questions, engage in discussions, give new ideas, and comment on articles.

I sincerely thank my pre-examiners Katariina Öörni and Hanna Jarva for their valuable critical comments.

I would like to acknowledge my collaborators Professor Matti Jauhiainen, whose enthusiasm has been a joy to watch; Professor Marja-Riitta Taskinen for her critical comments; and Docent Pirkko Pussinen as well as Professor Sohvi Hörkkö for good discussions.

I am further grateful to my colleagues and co-authors Aila Ahola, Christopher Fogarty, Josephine Järvelä, Daniel Gordin, Anmol Kumar, Ville-Petteri Mäkinen, and Lina Peräneva.

I also which to thank my fellow research scientists Emma, Niina, Nadja, and Bianca in our office for the warm and friendly atmosphere they have created, which has included interesting discussions on a wide range of topics. Further, Jenny, Johan, Jani, Iiro, Erkka, Maija, Hanna, Ninni, Nanna, Leena, Raija, Maija, and Valma have all contributed to the good feeling in our research group and have been ready to help, for which I am grateful.

This thesis would never exist without the nurses and laboratory technicians in FinnDiane. Tuula Soppela has been a friend and an invaluable support, both inside and outside of scientific matters. Anna Sandelin, Jaana Tuomikangas, and Mira Rahkonen have been vital in patient recruitment and investigation. Maikki Parkkonen is warmly acknowledged for her invaluable support in laboratory questions. Anna-Reetta, Mari, and Nanne are all thanked for their lunch break friendship as well as technical assistance.

Many thanks go to my friends Jenny, Emma, Anna, Minja, Milja, and Julia, who made studying biology fun and whose friendship and support have been invaluable along the way.

Finally, all the participants in the studies are acknowledged, without whom we would not be here today. Substantial financial support was provided by the Folkhälsan Research Foundation, the Stockmann Foundation, the Frenckell Foundation, Diabetestutkimussäätiö, the Novo Nordisk Foundation, the Lehikoinen Foundation, the Liv och Hälsa Foundation, and Svenska Kulturfonden.

I would like to thank my parents Kirsti and Steffe for their endless love and support in all aspects of life. You have always believed a PhD is worth pursuing and, more importantly, have been ready to wake up at sunrise with your grandchildren. My sister Helena is also warmly acknowledged; she has been a friend and supporter throughout my life.

Finally, I owe my warmest thanks to Toffe for always helping and supporting me. I really enjoy our family and everyday life with you.

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Helsinki 2016 ISSN 2342-3161 ISBN 978-951-51-2103-5